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Since the establishment of the more tumor susceptible SENCAR stock of mice, it has been used extensively in carcinogenesis assays by government agencies, academia, and commercial laboratories. The model has facilitated substantial cancer research involving mechanistic studies, as well as testing of various classes of chemicals, and complex mixtures such as organic extracts of drinking water, automobile exhaust, and consumer products, all of which are of particular concern to the regulatory agencies.

The purpose of this EPA Workshop on the SENCAR Mouse in Toxicological Testing is to provide a forum for the discussion of research data related to the use of this animal model. The workshop critically addresses the performance of the SENCAR mosue as a carcinogenesis assay model. Presentations of data by noted researchers deal with three categories relevant to the response of this mouse in laboratory tests: (1) Response to carcinogens—including strain and stock comparison and methodology for statisical analysis of mouse skin assays; (2) Alternate testing in the SENCAR—including in vitro comparison of SENCAR and BALB/c primary epidermal cells, metabolic activity in embryo cells, distribution studies, and modified initiation/promotion studies; and (3) Histopathology of the SENCAR mouse—including treatment-induced and spontaneous lesions, chronic effect of promoters, and pathology of aging SENCARs. The workshop concluded with an afternoon devoted to a round table discussion involving all presenters as well as members from the audience. A summary of this discussion follows the individual papers in these proceedings.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory, Research Triangle Park, NC and Cincinnati, OH, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. Wide ranges of pollutants known or suspected to cause health problems are studied. The research focuses on air pollutants, water pollutants, toxic substances, hazardous wastes, pesticides, and non-ionizing radiation. The laboratory participates in the development and revision of air and water quality criteria and health assessment documents on pollutants for which regulatory actions are being considered. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

The workshop critically addresses the performance of the SENCAR mouse as a carcinogenesis assay model. Presentations of data by noted researchers deal with three categories relevant to the response of this mouse in laboratory tests: (1) Response to carcinogens--including strain and stock comparison and methodology for statistical analysis of mouse skin assays; (2) Alternate testing in the SENCAR--including *in vitro* comparison of SENCAR and BALB/c primary epidermal cells, metabolic activity in embryo cells, distribution studies, and modified initiation/promotion studies; and (3) Histopathology of the SENCAR mouse--including treatment-induced and spontaneous lesions, chronic effect of promoters, and pathology of aging SENCARs.

F. Gordon Hueter, Ph.D.
Director
Health Effects Research Laboratory

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INTRODUCTION

by: Merrel Robinson*

The most widely accepted concept of tumorigenesis is a multistage process. The mouse skin has been frequently utilized for the experimental production of tumors. This model has been used to demonstrate both cocarcinogenesis wherein concurrent applications of causative agents result in tumor incidence while treatment with the agents singly does not and the initiation-promotion (two stage) model which classically involves a tumor initiating event followed in time by tumor promotion by an agent, which when applied repeatedly to the same area of skin, modifies the initiated cell and its environ so as to permit expression of the altered genetic structure. The promoter when applied alone causes only a few if any tumors. Excellent reviews of the multistage process of tumorigenesis are in print (1, 2, 3, 4, 5, 6).

As evidence of this mode of cancer induction has accumulated, the importance of identifying carcinogenic cofactors in the environment has become quite clear. The availability of dependable animal models for laboratory testing is of significant benefit in determining the carcinogenic potential of environmental contaminants. Historically the mouse has been the laboratory animal most susceptible to skin carcinogenesis by either a complete carcinogenesis or initiation-promotion protocol (7,8). The relative sensitivity of various stocks and strains of mice in these two protocols is SENCAR > CD-1 > C57Bl > BALB/c > ICR/Ha Swiss > C3H > DBA/2 (9).

Since the establishment of the more sensitive SENCAR stock of mice it has been used extensively in carcinogenesis assays by government agencies, academia, and commercial laboratories. The model has facilitated substantial cancer research involving mechanistic studies, as well as testing of various classes of chemicals, and complex mixtures such as organic extracts of drinking water, automobile exhaust and consumer products, all of which are of particular concern to regulatory agencies.

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METHODOLOGY FOR STATISTICAL ANALYSIS OF SENCAR MOUSE SKIN ASSAY DATA

by

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Key Words: Statistics, Tumor Analysis, Time to Tumor, Tumor Multiplicity, Survival Analysis, Nonparametric Statistics, Bioassay

Abstract

Various response measures and statistical methods appropriate for the analysis of data collected in the sencar mouse skin assay are examined. The characteristics of the tumor response data do not readily lend themselves to the classical methods for hypothesis testing. The advantages and limitations of conventional methods of anlaysis and methods recommended in the literature are discussed. Several alternative response measures are described which were developed specifically to answer the problems inherent in the data collected in the sencar bioassay system. These measures take into account animal survival, tumor multiplicity and tumor regression. Statistical methods for the analysis of these measures, testing for a positive dose response and a dose response relationship, are discussed. Sample data from representative initiation/promotion studies are used to compare the response measures and methods of analysis.

Introduction

The sencar mouse skin initiation/promotion bioassay has been widely used to test for chemical carcinogens and tumor initiators and promoters. A general description of the sencar skin assay system and of mouse skin tumorigenesis can be found in Slaga et al. and Bull, et al. (1,2). In general, the test substance is administered to the animals and the appearance of skin tumors (papillomas or carcinomas) on individual animals is charted for a specified period of time. Compounds, dose levels of a given compound and/or routes of exposure are then compared by evaluating the tumor incidence among the treatment groups of interest. A positive association is defined as a reproducible increase in the occurrence of tumors and a negative association as the absence of such an increase.

Statistics provide the necessary procedures for summarizing the relevant data and a mechanism for evaluating the strength of the association observed. As in any experimental situation, appropriate statistical methods are essential. The statistical analysis methods should be sensitive to the characteristics of the response data. Unfortunately, the characteristics of the sencar skin bioassay data do not lend themselves readily to the classicial techniques for data analysis. The multiplicity of tumors as well as their time of occurrence can and should be used as indicators of carcinogenicity.

The objective of this paper is to define appropr[illegible] sensitive and useful statistical procedures for analyzing data o[illegible] from a sencar mouse skin assay. Statistical methods commonly used or recommended in the literature will be reviewed. The advantages and disadvantages of each method will be discussed. Several alternative methods developed for the sencar tracking system at the Health Effects Research Laboratory of the U.S. Environmental Protection Agency will be presented and evaluated using representative data sets.

Several general principles were used as guidelines in the examination and evaluation of statistical methods for the analysis of sencar skin tumor data. They can be described as follows. The questions investigated by the experiment should be addressed by the test procedure in a straight forward manner with sufficient power to detect biologically meaningful differences. That is the rates of false positives and false negatives should be minimized by the methodology. The methods should be intuitively reasonable and sensitive to the characteristics of the data using all relevant information available. The assumptions for the validity of the statistical test must be reasonably met and the procedure should be robust, whether it is an omnibus test or a directional test.

Background

The sencar mouse skin assay can be designed to investigate two separate but related hypotheses. The first investigates a positive dose response and can be stated as follows: Is there evidence for an increase in tumor occurrence associated with the treatment? If there is then the second hypothesis investigates evidence for and may even estimate a dose-response relationship. The current work limits this investigation to a monotone response and thus the hypotheses can be stated as: Is there an increase in tumor occurrence associated with an increase in dose of a compound? Additionally, questions concerning the effect of route of exposure and tumorigenic potency can be addressed using the same methods discussed below. The experimental endpoints upon which statistical analysis is based include animal body weight, consumption data, tumor counts and pathology reports. Although each of these measurements supply information on toxicity, this paper will focus only on tumor count data and the problem of how to use it to calculate a reliable response measure for toxicity.

Methods

There have been several reports in the literature on the use of various statistical methods to analyze mouse skin assay data (3-6). The conventional approach to summarizing the assay results usually includes some or all of the information listed in Table 1. The classical statistical methods (eg. Student's t-test, contingency table analysis) are not necessarily appropriate for these response measures. The selection of a response measure and statistical methods for the analysis of tumor data is complicated by certain characteristics of the study data. Potential problems in the analysis arise from animal deaths prior to the termination of the study, differences in survival among the treatment groups, and tumor regression. Analysis of tumor occurrence is complicated by the incomplete observations in animals, which may be directly related to the toxicity of the compound. Tests which ignore these factors may result in a loss of power, that is the ability to detect true differences. If no early deaths occur, the data analysis is decidedly easier. Ignoring early animal deaths, and examining only surviving animals may lead to erroneous results.

The percent of animals with skin tumors at a specific time has been traditionally analyzed using Fisher's Exact Test for pairwise comparisons between treatment groups or a chi-squared test. In addition, linear, probit and logit models have been used to fit dose-response curves.

Tumor counts observed at a specific time have been analyzed using various parametric and non-parametric procedures. These include Student's t-test, analysis of variance procedures, linear regression, and log linear models. Drinkwater and Klotz (4) examined the analysis of tumor multiplicity data for surviving animals using the Student's t-test, Wilcoxon's Rank Sum Test and a two sample likelihood ratio test based on the negative binominal distribution. Both of these response measures ignore differential survival and provided no information on the pattern of tumor development. The parametric methods require various assumptions for their validity which may or may not hold true.

The pattern of tumor development can be examined by using the time to first tumor as the response measure. The time to tumor is calculated as the time from initial exposure to the appearance of the tumor. Cumulative incidence curves can be estimated using various parametric procedures, eg. Weibull (5), and non-parametric methods eg. Kaplan-Meier (3). The cumulative incidence curves can be graphically displayed and the estimated median time to first tumor reported as a descriptive statistic. These methods include all animals at risk, adjusting for differences in mortality rates among the groups. They provide overall measures of the compound's effect on initial tumor development. The entire incidence curve, rather than a single point in time can be statistically compared for differences among treatment groups. This method is limited by the fact that it ignores tumor multiplicity and the median time to first tumor cannot always be calculated directly from the observed data. Additionally, the parametric methods require the assumption of a specified model distribution which may or may not be tested for goodness-of-fit.

At the Toxicology and Microbiology Division of the Health Effects

Research Laboratory of the U.S. Environmental Protection Agency a tracking system has been developed and implemented for the collection of data in the mouse skin assay system. In addition to the daily observation of the animals for health monitoring and morbidity, a weekly observation of all animals on study is made and all grossly observed tumors are individually charted as to location, size and type (papilloma or carcinoma). Only those tumors that are observed for a minimum of three consecutive weeks are included in the permanent and cumulative tumor count. All individual tumors are charted weekly until they regress, coalesce or the animal is sacrificed.

In developing the methodology to be used in the analysis of tumor data from the tracking system, the multiplicity of tumors as well as their time of occurrence was used as indicators of carcinogenicity. The objective was to define summary response measures and statistical methods that would take into account simultaneously survival rates and the total tumor occurrence pattern, including tumor multiplicity and regression. Crump and Ng (7) developed several approaches for testing for a dose-related effect and applied the methods to several tracking system data sets. Table 2 lists the response measures considered. The response is calculated over the entire study time of interest. A response measure is calculated for each animal with its value censored at the time of the animals death. Tumors that appear at the same location at non-overlapping times are counted as single tumors.

The univariate response measures admittedly have inherent weaknesses. The time to first tumor is defined as previously discussed. The total number of permanent and cumulative tumors reflect tumor multiplicity, but do not take the time of occurrence and duration into account. The integral with respect to time of the number of tumors is defined as follows:

> Let $t_1<\ldots<t_k$ be the observation times. A tumor seen at time t_i, for $i = 2,\ldots,k-1$, contributes a factor of $(t_{i+1} - t_{i-1})/2$ to the integral. A tumor seen at time t_1 and t_k contributes a factor of $(t_2 - t_1)$ and $(t_k - t_{k-1})$, respectively to the integral. That is the integral is defined as
>
> $$n_1(t_2 - t_1) + \sum_{i=2}^{k-1} n_i(t_{i+1} - t_{i-1})/2 + n_k(t_k - t_{k-1}),$$

where n_i is the number of tumors seen at time t_i, for $i = 1,\ldots,k$. The integral takes duration into account but not the time of occurrence. The weighted sum of tumors weights each tumor by the time from its appearance until the end of the experiment.

To test for a dose response effect using the univariate response measures both trend and multiple comparison "survival" type analysis tests were examined by Crump and Ng. The multiple comparison test was that of Gehan and Wilcoxon (8,9,10). It is an omnibus test in the sense that it has power to detect nearly all types of differences. The trend test considered was a Cox-type trend test discussed by Tarone (11). It is based upon the proportional hazard model and is a one-sided directional test. For the univariate tests, it was assumed that the response measure, either the total number of tumors or the integral with respect to time of the number of tumors, was censored at the value attained at the time of death or study termination. The test statistics were calculated using the PHGLM and SURVTEST procedures from the Statistical Analysis System (SAS). The multivariate regression analysis method of Prentice et al. is a generalization of the Cox-type trend test applied to the univariate data. The multivariate test statistic was calculated using a modified version of the program R-COX developed at the Fred Hutchison Cancer Research Center.

Analysis of Sencar Tracking System Data

Sencar skin assay data collected on the tracking system were analyzed using the methods developed. The various response measures and statistical methods were compared. Table 3 describes the studies included in the present report. The evaluation included twenty-four data sets from initiation/promotion studies which followed, in general, the protocol procedure outlined in Bull et al. (2). The univariate response measures were calculated for two study duration times; twenty-four weeks and the entire study period.

Results of the Gehan-Wilcoxon multiple comparison test were compared to the Cox-type trend test for the analyses of the univariate measures (Table 4). The probability level of the test statistic was the basis for comparison. Using an α level of 0.05, the data sets were categorized according to the agreement of the results from the two test methods. For all response measures and time periods, the Gehan Wilcoxon test showed greater power that the Cox-type trend test. The difference between the methods was less in the time to first tumor analysis than in the integral or total tumor count analyses. In every analysis of tumor count data where the results differed between the two test methods, the Gehan-Wilcoxon test had a higher significance level. This is probably due to the fact that it tests for a wide range of alternative hypotheses, where as the trend test is testing for a monotone dose response.

The univariate response measures were compared based on the significance level of their test statistic using the Gehan-Wilcoxon method (Table 5). An α level of 0.05 was used as the cut point for significance. For both the twenty-four week data analysis and the entire study period analysis the percent agreement between response measures was at least ninety percent.

Eleven of the twenty-four data sets were analyzed using the multivariate stratified proportional hazard model of Prentice et al. In eight of the eleven cases, the results agreed with that of the univariate analysis. In two the three cases where there was disagreement, the multivariate test statistic showed a significant association between dose of the compound and tumor occurrence.

The univariate methods of analysis for the tumor count integral and total tumor count assumes that an animal's tumor observation is censored at its value at the time of death. This approach could possibly bias the analysis and lead to erroneous results. With both measures, animals dying at different times with equal responses are treated equivalently. Intuitively tumor free deaths at the end of the study should be greater evidence for no treatment effect than those earlier in the study. While death early in the study with a specific tumor response should be greater evidence for a toxic effect than an animal dying later with the same response. To examine the effect of mortality, the study time periods for data analysis were varied. The maximum difference (minimum test statistic p-value) in tumor occurrence was observed by twenty-eight weeks in the majority of the data sets analyzed (Table 6).

Discussion

Statistical methods supply the necessary procedures to quantify the strength of the evidence in support of the hypotheses under study. Thus, the methods used should be intuitively reasonable, easily interpretable, with sufficient power to detect meaningful differences. The advantages and disadvantages of various response measures and statistical methods for hypothesis testing of mouse skin assay data have been examined. Traditional methods which ignore survival differences, multiplicity of tumors and the pattern of tumor development may result in a loss of statistical power. Each method has its own deficiencies. None individually are sufficient to reliably describe and test assay data for toxicity. For example, unadjusted tumor incidence data should only be interpreted in conjunction with an examination of the survival patterns in the treatment groups. Methods which account for these factors eliminate biases and thus aid in ensuring the validity of the test results. Adjustment for these factors would be especially important for less potent compounds or studies based on low doses. The exact consequence of ignoring the potential analyses problems would depend on the characteristics of the data set in question.

Response measures and methods of analysis were derived for use in testing the toxicity of a compound when survival data, tumor count data and time of tumor occurrence are available. Only nonparametric and semiparametric techniques were considered to eliminate the necessity of specifying a response distribution. The methods developed are advantageous for several reasons. The analyses include every animal not just animals surviving to a given time. Tumor multiplicity is accounted for by each of the response measures and the duration and regression of tumors by several of the univariate methods. The multivariate response measure considered is the only measure examined that is not biased by any of the characteristics of the data. It accounts for mortality and time of tumor occurrence. While intuitively, this method seems the most reasonable, it has the disadvantage in that several assumptions are necessary for its validity. The stratified proportional hazard model assumes that the hazard of the dose groups is proportional to that of the control group and that tumors occur in a single animal as a non-homogenous Poisson process. The model does not allow for extra variably to account for animal to animal variation in susceptibilities (7). Additionally, this method is the most difficult computationally in that the computer software is not readily available.

Each of the univariate response measures have limitations and thus, the analysis of the mouse skin assay data should not be confined to one of these measures. A thorough analysis should include an examination and test for equality of survival distributions, a comparison of time to first tumor distributions and a comparison of tumor multiplicity. Each of these measures contain important information that should be used to interpret the results of an experiment. Although there were relatively few cases in which the analysis of the integral and total tumor count response measures differed, the integral accounts for tumor duration and thus is preferrable to the total tumor counts as a response measure. The strong performance of the Gehan-Wilcoxon multiple comparison test in the analysis to the sencar tracking system data leads to the recommendation of its useage. As a result of these analyses, if an association between tumor response and dose of a compound is found and it is desirable to examine the data for a monotone dose response, a trend analysis using the

Cox-type trend test can be performed. By conducting the analysis in the manner, full use is made of all available information on the toxicity of a compound.

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Table 1. Summary Report Information

Summary Reports Generally Include the Following:
Dose
Number of Animals Exposed
Response Measures
Percent of Animals with Tumors
Average (Median) Number of Weeks to 1st Tumor (±S.D.)
Time to 1st Tumor
Time to Last Tumor
Number of Tumors per Animal

Table 2. Tumor Response Measures

Measure	Comments
Univariate:	
Time to 1st Tumor	Ignores Multiplicity of Tumors
Total Number of Permanent Tumors	Ignores Time of Tumor Occurrence, Duration and Tumor Regression
Total Number of "Cummulative" Tumors	Ignores Time of Tumor Occurrence and Duration
Integral w.r.t. time of the Number of Tumors	Takes Duration Into Account But Ignores Time of Occurence
Weighted Sum of Tumors	Each Tumor Weighted by Its Time of Appearance. Ignores Tumor Regression
Multivariate:	
Multiple Times to Tumor	Takes Multiple Tumors Per Animal Into Consideration

Table 3. Tumor Multiplicity Data

No. of Studies	No. of Dose Groups	No. of Animals Per Group	PMA	Length of Study (Weeks)
8	2	40	yes	31 - 36
10	3	40	yes	52
6	4	40	yes	52

[a]Tumor Type - Skin papillomas and carcinomas

Mouse Strain - Sencar

Only those tumors observed for 3 consecutive weeks are included in tumor count

Table 4. Gehan-Wilcoxon Multiple Comparison Test
vs.
Cox-Type Trend

Response Measure	Total No. of Data Sets	Percent of Data Sets GW<Cox[d]	Percent of Data Sets GW>Cox
Time to 1st Tumor			
+/+[a]	15	60	40
-/-[b]	8	63	37
+/-[c]	1	0	100
Integral w.r.t. time (week = 24)			
+/+	7	86	0
-/-	8	100	0
+/-	5	100	0
Integral w.r.t. time (week = last)			
+/+	8	75	25
-/-	9	78	11
+/-	7	100	0
Total No. Permanent Tumors (week = 24)			
+/+	7	100	0
-/-	9	89	11
+/-	4	100	0
Total No. Permanent Tumors (week = last)			
+/+	12	92	8
-/-	9	78	22
+/-	3	100	0
Total No. Cummulative Tumors (week = 24)			
+/+	7	100	0
-/-	8	100	0
+/-	5	100	0
Total No. Cummulative Tumors (week = last)			
+/+	7	86	14
-/-	9	89	11
+/-	4	100	0

---

[a] significant ($p \leq 0.05$) by both statistical methods of analysis
[b] non-significant ($p > 0.05$) by both statistical methods of analysis
[c] significant by only one statistical method
[d] Comparison based on test statistic p-value, tied values omitted

Table 5. Comparison of Response Measures

Univariate

	Percent Agreement[a]	
	24 Week Data	Last Week Data
Time to 1st Tumor vs.		
Integral w.r.t. Time	95	92
Total No. Permanent Tumors	90	92
Total No. Cummulative Tumors	95	90
Integral w.r.t. Time vs.		
Total No. Permanent Tumors	95	100
Total No. Cummulative Tumors	100	100
Total No. Permanent Tumors vs.		
Total No. Cummulative Tumors	95	100

Multivariate

Agreed with univariate results in 8 of the 11 data sets analyzed.

Multivariate found significant differences in 2 of the 3 analyses for which there was disagreement.

---

[a] Based on Gehan-Wilcoxon Test Statistic p-value ≤ 0.05 vs. p-value >0.05

Table 6. Effect of Early Cut-Off Time

	Number of Studies				
	Week[b]				
Response Measure	24	28	32	>32	Total
Integral w.r.t. time					
Significant[a]	11	3	1	3	18
Non-significant	3	-	-	3	6
Total No. Current Tumors					
Significant	10	4	1	2	17
Non-significant	3	-	-	4	7
Total No. Cummulative Tumors					
Significant	9	4	-	-	13
Non-significant	3	-	-	4	7

[a] $p \leq 0.05$ Gehan-Wilcoxon Multiple Comparison Test
[b] Earliest week minimum p-value obtained (ie. maximum difference observed)

ASSOCIATION OF CARCINOMA YIELD WITH EARLY PAPILLOMA DEVELOPMENT IN SENCAR MICE

by

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ABSTRACT

The responsiveness of the SENCAR mouse skin to 20 different chemicals with known carcinogenic properties was assessed in initiation/promotion experiments. The purpose of these experiments was to 1.) evaluate the extent of false negative responses in mouse skin initiation/promotion protocols and 2.) determine the extent to which early papilloma development can be used to predict the eventual development of malignant tumors. The chemicals were administered as initiators by four different routes; orally, intraperitoneally, subcutaneously and topically. Following the initiating dose of carcinogen, the animals were subjected to triweekly, topical applications of 1 ug 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for a period of twenty weeks. The yield of papillomas at 24 weeks was selected as a potential predictor of carcinoma yields at 52 weeks following the start of the promotion schedule. Positive responses were observed with only eight of the compounds tested. Where positive results were observed there was some evidence that the response could depend both qualitatively and quantitatively upon the route of administration. However, no route was clearly superior in that different chemicals gave greater responses by different routes. Papilloma yield at 24 weeks following the start of the promotion schedule was clearly related to the development of carcinomas at 52 weeks. There was not a simple linear relationship between papilloma yield and carcinoma development in that the number of malignant tumors per papilloma decreased with increasing papilloma yields. The relationship between papilloma and carcinoma yields appeared to be independent of the carcinogen employed. These data indicate that there are some limitations in the use of mouse skin initiation/promotion experiments as the sole basis of identifying substances with carcinogenic activity. However, the test does perform well within certain classes of compounds. Within the limits of these chemical classes, the use of papilloma yields to predict carcinoma yield appears to be justified.

INTRODUCTION

There are a variety of reasons for pursuing animal models that display a high degree of responsiveness to particular stimuli. From the point of view of fundamental research a strain or stock of animals that is particularly susceptible to a particular disease state provides a tool for identifying some of the key steps involved in the development of the disease and thus provides insights into the treatment and/or prevention of the disease. From the context of an agency that is responsible for insuring that dangerous chemicals are not introduced into the environment, a sensitive strain provides the possibility of simplifying testing schemes.

Our interest in the SENCAR stock was two-fold; 1.) whether the increased sensitivity of the skin in this stock would broaden the classes of chemical carcinogens that could be detected and 2.) the extent to which papilloma development could be used to predict the ultimate incidence of malignant tumors. If these two questions could be answered in the affirmative, it would allow a much broader use of the skin tumorigenesis model and its inherit convenience as a short-term test method in environmental carcinogenesis. Our particular hope was that we could take advantage of the rapid development of papillomas that results in the initiation/promotion protocols which employ 12-O-tetradecanoyl-phorbol-13-acetate (TPA) as a tumor promoter. The short time involved in the development of these tumors and the fact that they are an external lesions that are easily identified would make SENCAR mice an ideal system for routine testing of tumor-initiating agents.

To establish the extent to which SENCAR mice might fit this ideal we have subjected 20 different carcinogens, representing 14 different chemical classes, to tests as tumor initiators (Bull and Pereira, 1982). We also chose to explore the extent to which the route of administration influences the response to these chemicals in a classical initiation/promotion protocol.

MATERIALS AND METHODS

Chemicals.

All compounds tested as initiators were purchased at the highest purity available and submitted for analysis by a contract laboratory, Environmental Health Research and Testing, Inc. (Contract No.68-03-1720, M. Pereira, project officer). The results of these analyses and the source of each chemical is provided in Table 1. Only in the case of 2-acetylaminofluorene does it appear that the level of purity was so low as to call the results reported here into question. This compound was found to be only 50% pure. Since the experiment had essentially been completed before the analysis were completed, the results are included in this report. TPA was obtained from Chemical Carcinogenesis, Eden Prairie, MN.

Animals

Female SENCAR mice were used in all experiments. As indicated in Table 1, mice were obtained either from Oak Ridge National Laboratories, Oak Ridge, TN or from Harlan Sprague-Dawley, Indianapolis, IN. Animals were begun on study when 6-8 weeks of age and were maintained on Purina rodent chow (Ralston Purina Co., St. Louis, MO) and distilled water *ad libitum*.

Experimental.

Depending upon the intent of the experiment, each group of animals contained either 40 (first round screening), 30 or 25 mice (when multiple doses were utilized to confirm positive results and to establish dose-response relationships). The backs of the animals were shaved 3 days prior to application of the test chemical (tested as initiators, only) and once weekly during the tumor promotion period. Using available acute toxicity data, the initial doses used in the screening phase was selected to approximate 1/5 of the LD_{50}. With indications of a positive response additional doses were tested. On occasion, if the initial dose resulted in a marginal response, higher total doses were administered by splitting the initiating dose into 6 equal doses that were applied over a two week period. Depending upon the compound and the route of exposure either acetone, emulphor (a polyoxygenated vegetable oil), ethanol, saline or water was used as the vehicle for the test chemicals. Control groups received equivalent amounts of the appropriate vehicle.

Two weeks following the administration of the last dose of the test chemical, a promotion schedule was begun which involved the application of 1.0 ug TPA in 0.2 ml acetone to the back of the animal 3 X weekly. The topical route was always utilized for TPA administration irregardless of the route by which the test chemical was administered. At the highest dose utilized of each test chemical a group was included that received acetone applications in the place of TPA applications. These groups were usually limited to 20 animals and served to determine whether the single dose of the chemical could induce tumors in the absence of TPA promotion. In no case were significant numbers of tumors observed in these groups. Consequently, the results were omitted from this paper to simplify the presentation.

Tumor incidences were recorded weekly for the first 24 weeks following the start of promotion, and monthly thereafter. To be included in the cumulative count, a tumor had to be at least 1 mm in diameter and be present in the same location for 3 consecutive weeks. At 52 weeks the experiments were terminated and histological evaluations performed for all gross lesions that were noted at necropsy. Some experiments were terminated prior to 52 weeks when they were clearly negative (<0.1 tumor/animal) at all doses tested at 24 weeks.

RESULTS

Table 2 summarizes the data obtained in the testing of all chemicals examined in terms of positive or negative responses. Chemicals were judged positive if the response was significantly different from the concurrent control group (using a multiple cell contingency test based on the number of animals bearing tumors, at $P < 0.01$) and if a response of greater than 0.4 tumors/animal was observed in a group of 40 animals. A positive response is also indicated if the tumor yield was between 0.3 and 0.4 tumors/animal if the results were confirmed in a separate experiment involving multiple doses and the results increased the statistical confidence relative to the first experiment. Overall, the control response to TPA promotion in the absence of prior treatment with a chemical was 0.08 tumors per animal (total of 50 tumors in 622 animals). However, this value varied considerably between experiments and indicates the need for caution in accepting the statistical evaluation of a single experiment when a low-level response was observed.

The results in Table 2 indicate that eight of the twenty chemicals tested for their ability to initiate skin tumors in SENCAR mice were found to be positive by one or more routes of administration. Six of the eight positive compounds were found to initiate tumors when applied topically, the usual route of administration. Seven of the eight compounds were positive by the subcutaneous route (administered in the back) and five of the eight were positive if the chemical was administered orally.

When administered by the oral route, all three PAHs tested (7,12-dimethylbenzanthracene, benzo(a)pyrene and 3-methylcholanthrene), ethyl carbamate and methylnitrosourea were positive. The topical route of exposure added 2-propriolactone to the list of positive compounds. This was the only route by which 2-propriolactone produced a significant response. FANFT and azaserine were positive only if administered subcutaneously. Testing by the intraperitoneal route was limited to a few compounds and there were no uniquely positive or negative compounds identified by this route.

Figure 1 provides the dose-response information obtained for the eight positive compounds. The route-specific nature of the responses were not entirely qualitative, since rather marked differences in potency were observed using different routes of administration of the same chemical. PAHs, in particular, are very much more potent when they are topically applied relative to systemic routes of administration (Figure le, 1f and 1g). In general, the activity observed with PAHs by this route was at least

2 orders of magnitude greater than the alternate routes. On the other hand, ethyl carbamate was approximately two times as potent in initiating skin tumors by systemic routes of administration than by topical application. The basis for these differences was not clear, but may be related to the way in which the activated form of the chemical reached its critical site of action.

In general, responses were observed to increase with the dose of initiating agent. This was particularly apparent with the PAHs where the multiplicity of tumors continued to increase to 10 or more tumors/animal as the dose was increased. At that point a decreased response was noted with DMBA. This decrease may be more apparent than real because of the difficulty of accurately scoring tumors as they exceed this number. On the other hand, the response to 2-propriolactone leveled off and even decreased slightly as the tumor yield began to exceed 3 tumors/animal. This apparently reflected a real maximum in the response to 2-propriolactone. Similar types of maxima have been observed with other direct acting initiators (Bull et al., 1985). The limitation on the response to FANFT appears to be much more severe than that observed with these other agents. Responses of similar magnitude were seen with subcutaneous administration of FANFT in other experiments and the data have repeatedly met our criteria for statistical significance. It is perhaps notable that FANFT was clearly negative by all other routes of administration. Therefore, the possibility that this response was secondary to local irritation produced by FANFT cannot be excluded. The only other compound that was positive only by the subcutaneous route, azaserine, produced a much less equivocal result and which did not appear to be self-limiting. One possible explanation is that the capacity for local metabolism of FANFT is limited to account for the limited response and that systemic metabolism produced metabolites that were not active in the skin.

The relationship between papilloma development at 24 weeks after the start of the promotion schedule (or 4 weeks following its termination) and the incidence of carcinomas observed in animals maintained on study for a full year is plotted in Figure 2a. Although the probability of carcinoma development increases as the yield of papillomas increases, it was clear that there is not a simple linear relationship between the development of benign and malignant tumors. Only data obtained from groups that averaged less than 14 papillomas/animal were included in the figure to minimize the problem of scoring coalesced tumors. Another reason for limiting the results to a maximum response was that the yeild of carcinomas in 52 weeks per papilloma at 24 weeks approximated 0.1. Higher doses resulted in earlier development of fatal malignant tumors. These early deaths obviously distort any simple relationship between the two tumor types at these two time intervals. An additional concern about the relationship between carcinoma development versus early papilloma yield was that the upper portions of the curve (i.e. greater than 2 papillomas/animal) is dominated by data from PAHs; no other chemical produced an average tumor yield of above 4 papillomas/animal. Consequently, the PAH data and the non-PAH data have been segregated in panels b and c of Figure 2. The curve has also been confined to tumor yields of less than 4/animal to allow a better comparison of the relationship at these lower tumor yields. There are no obvious differences in the relationship between papillomas and carcinoma whether they are induced by PAH or non-PAH carcinogens.

In Table 3 the control animals for those experiments that involved topical applications of the chemicals can be compared across individual experiments. In addition, the reproducibility of the responses of female SENCAR to identical doses of benzo(a)pyrene administered as the positive control in a variety of other experiments is also presented. All of these animals were subjected to the TPA promotion schedule. It is apparent from these data that there is considerable variability in the response of SENCAR mice to a constant dose of benzo(a)pyrene. However, the relationship between cumulative papilloma yield at 24 weeks and carcinoma development at 1 year remained within fairly narrow limits (i.e. a ratio of 0.07 to 0.12 carcinomas/papilloma) despite a 6-fold variation in the papilloma yield. Similar variation in response was observed in the papilloma yield in animals that received only TPA promotion (except for the application of the vehicle). In this case the control incidence of papillomas varied from zero to as high as 0.45/animal. Admittedly, the 0.45 value is very rarely encountered (this was only the second time in more than 100 experiments in our experience). However, it does point out the difficulty in accepting marginal responses as significant (e.g. our results with FANFT) without confirmation in separate experiments.

CONCLUSIONS

The present study was undertaken as part of a larger effort directed at the question of whether a group of short-term *in vivo* carcinogenesis assays might allow some estimation of carcinogenic hazards without resorting to life-time feeding studies (Bull and Pereira, 1982). Such an approach had be previously suggested by Weisburger and Williams (1977). The major reason for chosing multiple tests was because a cursory examination of the literature appeared to indicate that individual test systems based upon the responses of a single target organ had relatively high rates of false negative responses. This was definitely the case with the mouse skin initiation-promotion protocols that have utilized strains of mice less sensitive to tumor initiation than the SENCAR stock (Pereira, 1982).

Chemicals chosen to evaluate the various test systems (Bull and Pereira, 1982) were deliberately chosen to represent a wide variety of chemical classes and to impact a variety of target organs. In some respects the chemicals selected provided an overly rigorous test of each systems ability to respond. For example, although benzene has been recognized as a human carcinogen for years it has been very difficult to induce tumors in experimental animals with this chemical. Similarly, the carcinogenic activity of 1,4-dioxane is very low and questions concerning the mechanism by which lead acetate produces renal tumors poses some difficulties. However, it was felt as a first approximation that the definition of carcinogenic activity must simply refer to the ability to increase tumor tumor incidence in lifetime studies with no regard for potential differences in mechanism.

Despite the above reservations, it was clear that the rate of false negative responses to chemicals with recognized carcinogenic activity is quite high when they are tested as tumor initiators in the mouse skin. There can be no doubt about the carcinogenic activity associated with 2-acetylaminofluorene, aflatoxin B_1, azobenzene, dibromoethane, dibutylnitrosamine, and diethylnitrosamine. Overall the false negative rate was 60%.

These results do not argue that the mouse skin initiation/promotion assay is without value in evaluating carcinogenic activity of various chemicals. This system appears to be well adapted to the detection of a variety of direct acting carcinogens (Pereira, 1982; Bull et al., 1985). It has been routinely utilized in assaying the carcinogenic potency of PAHs (Slaga et al., 1982) and has been useful in detecting carcinogenic activity of chemicals possibly related to ethyl carbamate in terms of their mechanism of action such as vinyl carbamate and acrylamide (Bull et al., 1984).

From the present results it would appear that the development of papillomas was predictive of the later development of carcinomas. This was in essential agreement with the data reviewed by Burns et al. (1984) which indicated a 5 to 7 percent conversion of papillomas to carcinomas. To the extent that it could be ascertained, this relationship did not appear to be dependent upon the individual chemical. There was as much scatter in the relationship when a single chemical was involved (benzo(a)pyrene) in multiple tests (Figure 2b) as when a variety of chemicals were involved in the tests (Figure 2c). It should be noted, however, that there is not a simple linear relationship between the development of papillomas at 24 weeks and carcinoma incidence at 52 weeks. It was clear that circumstances involving low papilloma yields (including animals that received only TPA treatments) had a higher liklihood of yielding carcinomas relative to the papilloma yield than when high papilloma yields were involved. One must be cautious in interpeting this data because the lethality of malignant tumors places a bias in the results at high papilloma yields. Nevertheless this curvilinear relationship appeared to be present at papilloma yields where survival was not limiting (i.e. < 4 papillomas/animal).

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Table 1. Source and Analysis of Carcinogen Test Matrix Chemicals and Source of SENCAR Mice Used to Test Each Compound

CMPD	Source	Lot #	% Purity	Contaminants		SENCAR Source
2-Acetylaminoflourene	Sigma, St. Louis, MO	1092-0202	50			HSD
				Carbonic Acid	30%	
				2 Methoxy Benzoic Acid Methy Ester	14%	
				Triphenylmethane	4.1%	
Aflatoxins	Sigma	117C-0218	CND			OR
Azaserne	Sigma	72F-0401	99+			HSD
Azobenzene	Eastman, Rochester, NY	B7A	99+			OR
Benzo(a)Pyrene	Sigma	15C-0116	99+			OR
1,2-Dibromoethane	Fluka, Hauppague, NY	171756104	92	Bromochloroethane	5.0%	HSD
				Dibromopropane	0.3%	
				Tribromomethane	0.3%	
				Tribromoethane	1.7%	
Diethylnitrosoamine	Sigma	49C-0348	99+			OR
				1,2,3-Trichloropropene	0.2%	
1,2-Dimethylhydrazine	Aldrich, Milwaukee, WI	092497	98			HSD
1,4-Dioxane	Aldrich	101197	99+			HSD
Ethyl Carbamate (Urethane)	Aldrich	JD031997	99+			OR
Lead Acetate	Fisher, Cincinnati, OH	726062	99+			OR
3-Methylcholanthrene	Fluka	66230	99+			HSD
Methymethanesulfonate	Aldrich	4120TD	99			OR
N-[4-(5-nitro-2-furyl)-2-thiazoyl]-formanide (FANFT)	Saber Lab, Morton Grove, IL	791101	99+			OR
2-Napthylamine	Sigma	18C-0440-1	99			HSD
S-(2-propenyl)-1,3-benzodiazole (Safrole)	Aldrich	050197	90.2	Camphor	0.8%	HSD
				Cadinenes scans	4.9%	
2-Propiolactone	Sigma	109C-0516	96	2-oxo-Propanoic acid	1.5%	OR/HSD
				Acetic Acid Scans	2.4%	
2,4,6-Trichlorophenol	Eastman	B7B	99+			OR

CND - Could not determine
OR - Oak Ridge National Laboratory
HSD - Harlan Sprague-Dawley

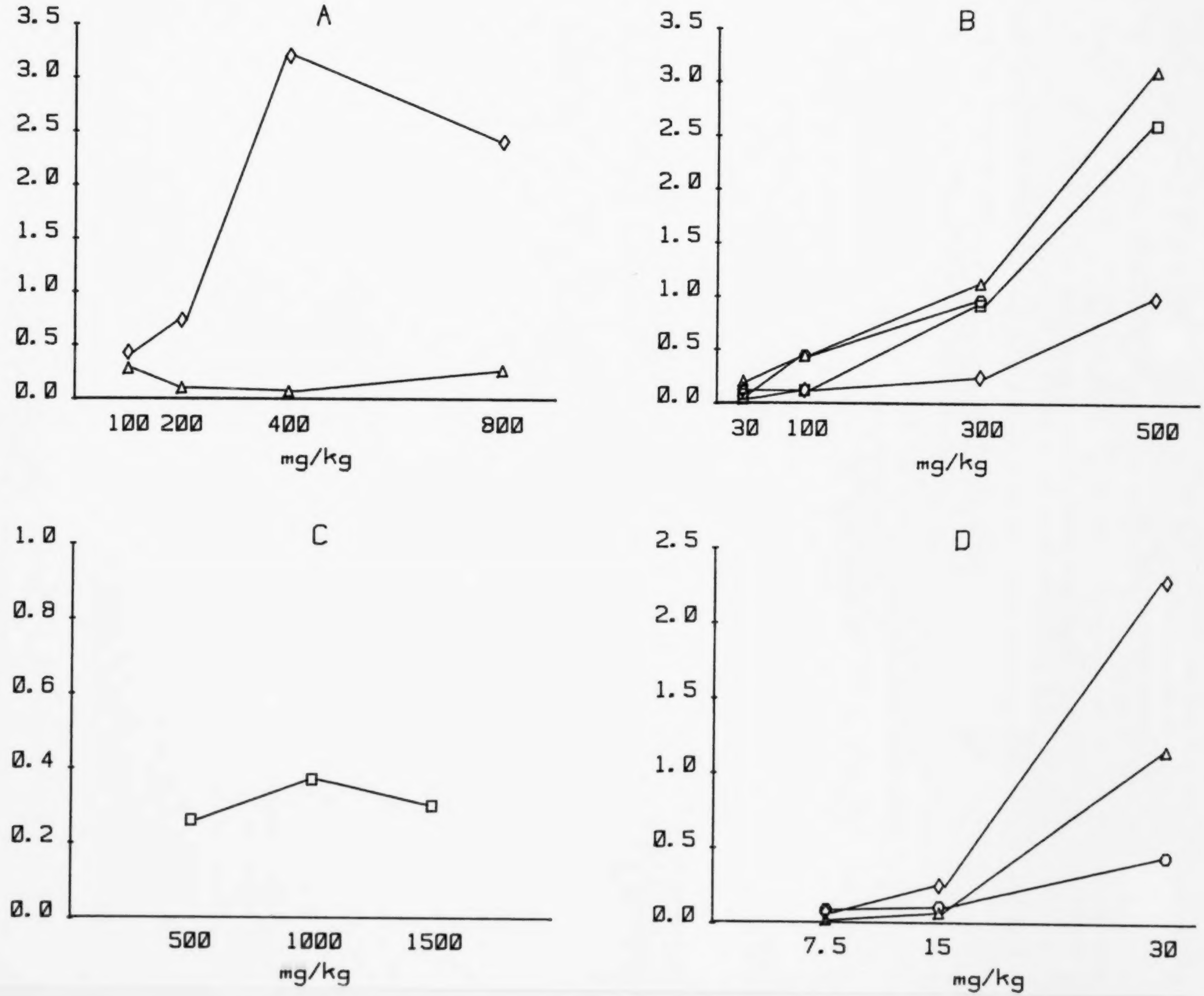
Table 2. Summary of Positive and Negative Results with Chemical Carcinogens Tested as Tumor Initiators in the Skin of Female SENCAR Mice by Different Routes of Administration.

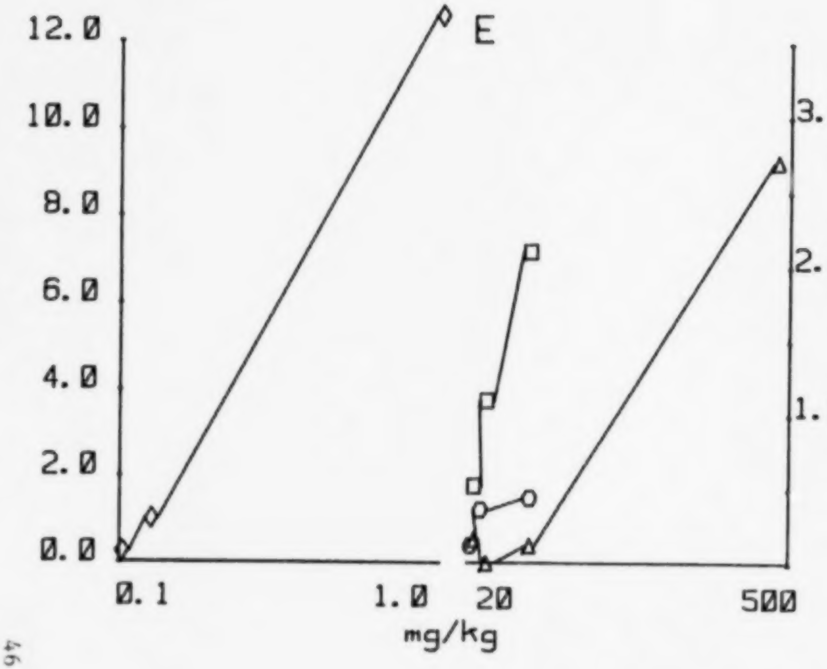
Compound	Dose Producing Largest Response (mg/kg)	Route of Exposure			
		Oral	IP	SC	Topical
2-Acetylaminofluorene	200	--	NT	--	--
Aflatoxin B_1	1.5	--	NT	--	--
Azobenzene	200	--	NT	--	--
Azaserine	30	--	NT	++	NT
Azaserine	200	NT	NT	NT	--
Benzene	800	--	NT	--	--
Benzo(a)pyrene	50	NT	NT	NT	++
Benzo(a)pyrene	100	NT	++	++	NT
Benzo(a)pyrene	500	++	NT	NT	NT
Dibromoethane	50	--	NT	--	NT
Dibromoethane	200	NT	NT	NT	--
Dibutylnitrosamine	200	--	NT	--	NT
Dibutylnitrosamine	400	NT	NT	NT	--
Diethylnitrosamine	50	--	NT	--	--
Dimethylbenzanthracene	1	NT	++	++	++
Dimethylbenzanthracene	5	++	NT	NT	NT
1,4-Dioxane	1000	--	NT	--	--
Ethyl carbamate	300	NT	++	NT	NT
Ethyl carbamate	500	++	NT	++	++
FANFT[a]	500	--	NT	NT	--
FANFT	1000	NT	NT	++	NT
Lead acetate	600	--	NT	NT	--
3-Methylcholanthrene	250	++	NT	++	NT
3-Methylcholanthrene	500	NT	NT	NT	++
Methylmethanesulfonate	40	--	NT	--	--
Methylnitrosourea	30	++	++	++	++
B-Propriolactone	100	--	NT	--	+-
B-Propriolactone	800	--	NT	NT	++
Safrole	470	--	NT	--	--
2,4,6-Trichlorophenol	200	--	--	--	--

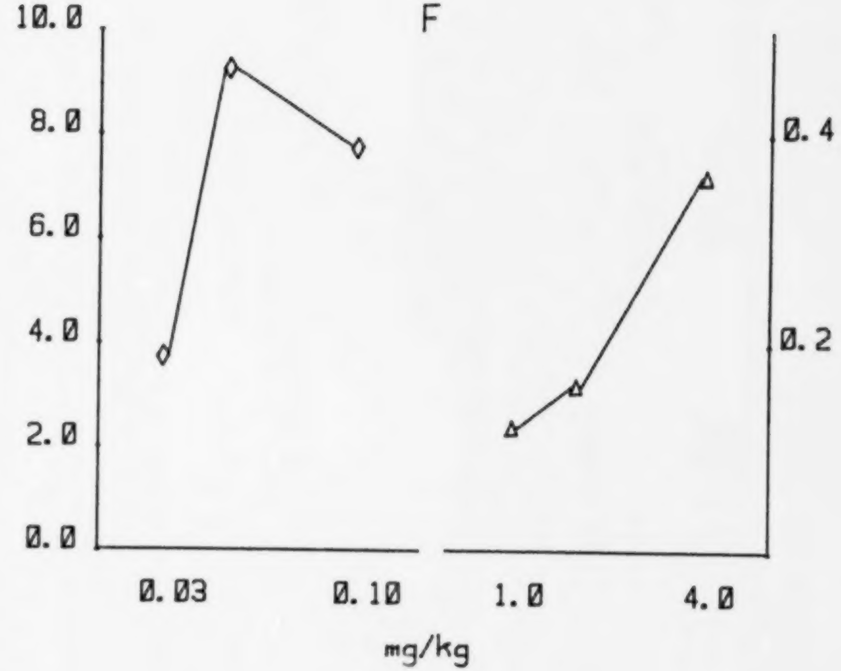
NT = Not tested, -- = Negative, ++ = Positive, +- = Equivocal

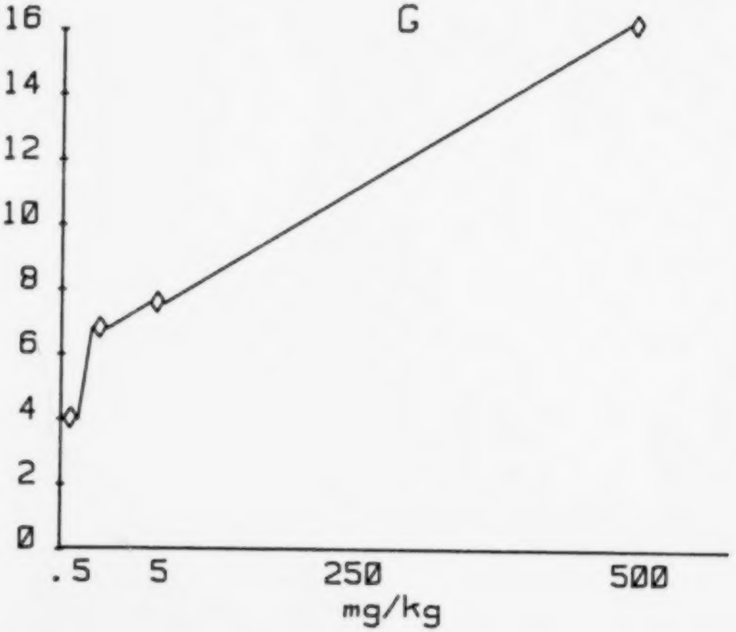
[a] = N-(4-(5-nitro-2-furyl)-2-thiazolyl)-formamide.

Figure 1. The dose-response relationships of chemicals that were found to be positive as tumor initiators in the skin of female SENCAR mice. Data plotted is the cumulative papilloma yield (expressed as average tumors per animal) observed 24 weeks after beginning the tumor promotion schedule with TPA. Panel A = B-propiolactone, B = ethyl carbamate, C = FANFT or N-(4-(5-nitro-2-furyl)-2-thiazolyl)-formamide, D = methylnitrosourea, E = benzo(a)pyrene, F = dimethylbenzanthracene, G = 3-methylcholanthrene, H = azaserine.


A
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100 200
400
800
mg/kg
B
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
30
100
300
500
mg/kg
C
1.0
0.8
0.6
0.4
0.2
0.0
500
1000
1500
mg/kg
D
2.5
2.0
1.5
1.0
0.5
0.0
7.5
15
30
mg/kg


E
12.0
10.0
8.0
6.0
4.0
2.0
0.0
3.0
2.0
1.0
0.1
1.0
20
500
mg/kg


F
10.0
8.0
6.0
4.0
2.0
0.0
0.4
0.2
0.03
0.10
1.0
4.0
mg/kg


G
16
14
12
10
8
6
4
2
0
.5
5
250
500
mg/kg

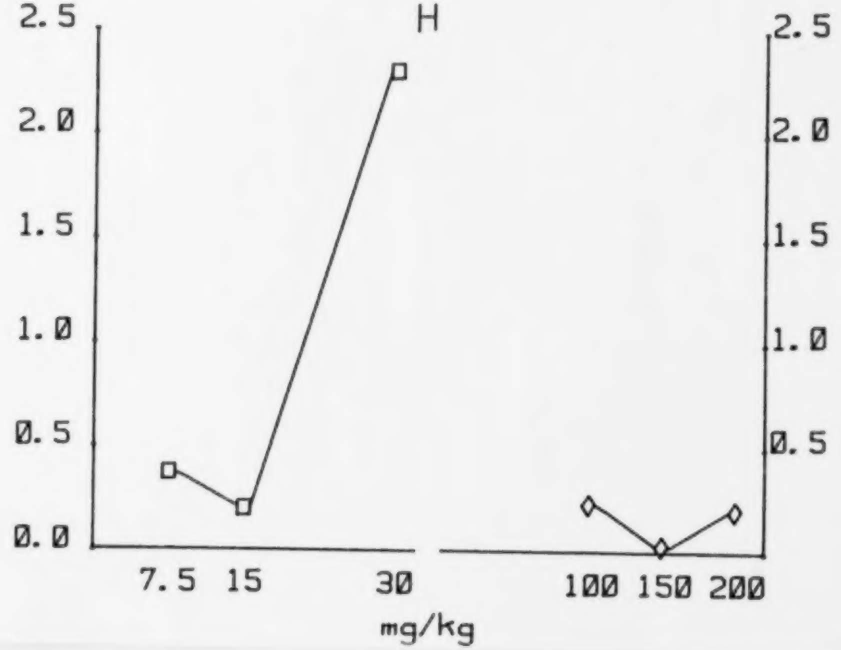
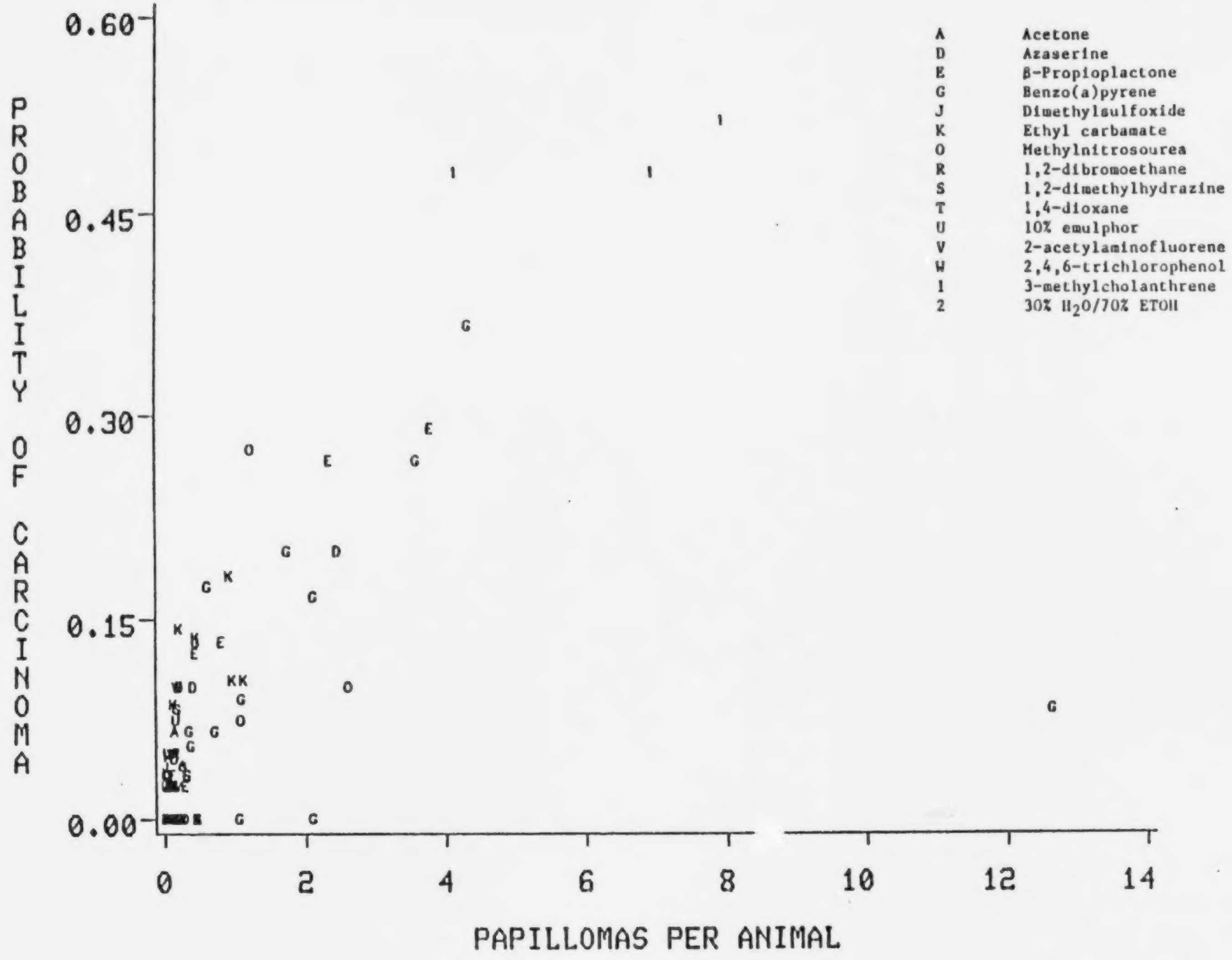

H
2.5
2.0
1.5
1.0
0.5
0.0
2.5
2.0
1.5
1.0
0.5
7.5
15
30
100
150
200
mg/kg

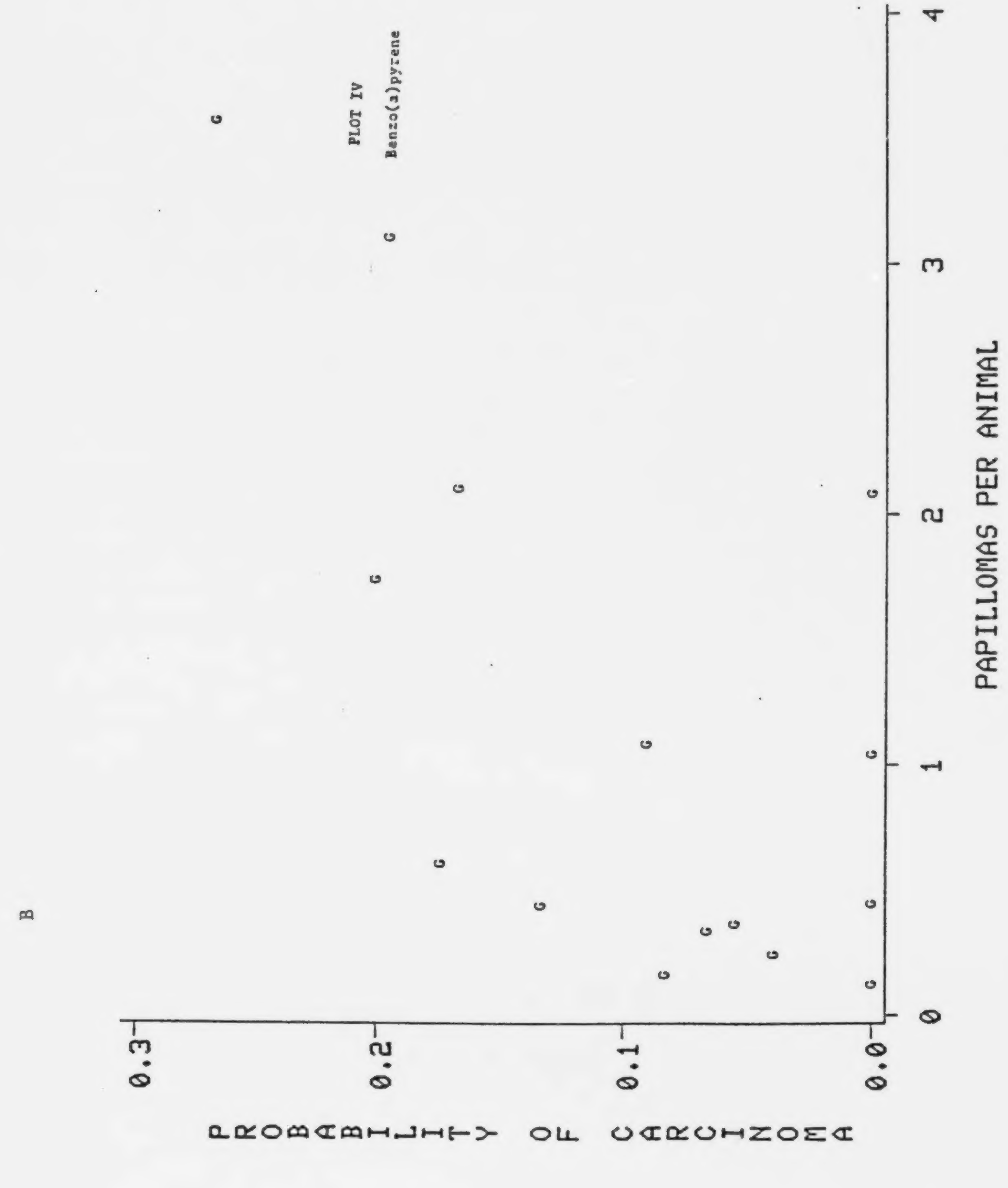
Figure 2. The relationship between papilloma yield at 24 weeks following the beginning of the tumor promotion schedule and the carcinoma yield by 52 weeks. Panel A includes all the data from experiments that were maintained for the 52 week time period. Panels B and C provide an expanded scale comparison of this relationship with benzo(a)pyrene (B) and non-PAH initiators (C).

A



PLOT II

A Acetone
D Azaserine
E β-Propioplactone
G Benzo(a)pyrene
J Dimethylsulfoxide
K Ethyl carbamate
O Methylnitrosourea
R 1,2-dibromoethane
S 1,2-dimethylhydrazine
T 1,4-dioxane
U 10% emulphor
V 2-acetylaminofluorene
W 2,4,6-trichlorophenol
1 3-methylcholanthrene
2 30% H2O/70% ETOH
PROBABILITY OF CARCINOMA
0.60
0.45
0.30
0.15
0.00
PAPILLOMAS PER ANIMAL
0
2
4
6
8
10
12
14


PLOT IV
Benzo(a)pyrene
PROBABILITY OF CARCINOMA
0.0
0.1
0.2
0.3
PAPILLOMAS PER ANIMAL
0
1
2
3
4
B
G

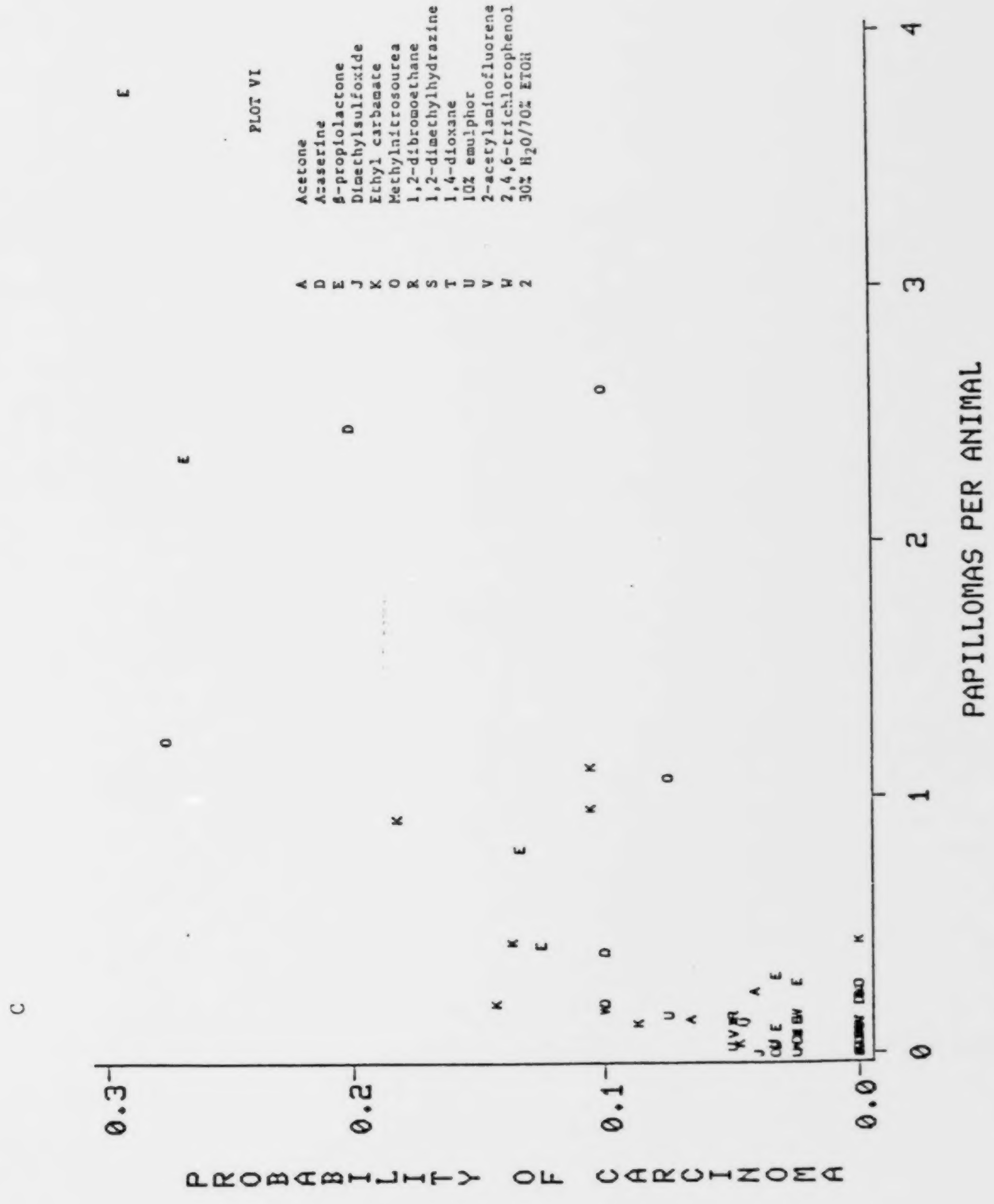

PLOT VI
A Acetone
D Azaserine
E β-propiolactone
J Dimethylsulfoxide
K Ethyl carbamate
O Methylnitrosourea
R 1,2-dibromoethane
S 1,2-dimethylhydrazine
T 1,4-dioxane
U 10% emulphor
V 2-acetylaminofluorene
W 2,4,6-trichlorophenol
Z 30% H_2O/70% ETOH
PROBABILITY OF CARCINOMA
0.0
0.1
0.2
0.3
PAPILLOMAS PER ANIMAL
0
1
2
3
4

Table 3. Reproducibility of Tumor Yields in Control and Benzo(a)pyrene-Initiated Mice Following TPA Promotion.

Treatment		Trial 1	2	3	4	5	6	7	Sum Avg
Benzo(a)pyrene[a]	N	29	29	15	29	30			132
	Paps./mouse[b]	3.48	4.44	1.73	0.72	2.10			2.58
	Carcin./mouse[c]	0.27	0.37	0.20	0.07	0.07			0.22
	Carcin./Paps.	0.07	0.09	0.12	0.10	0.08			0.085
Acetone	N	29	40	40	40	40	30	24	243
	Paps./mouse	0.14	0.08	0.23	0.45	0.03	0.03	0.00	0.15

---

[a] Benzo(a)pyrene was administered topically at a dose of 1 mg/kg body weight. In all cases, 1 mg TPA was applied topically in 0.2 ml acetone 3 times weekly beginning 2 weeks after the initiating dose and continued for 20 additional weeks.

[b] Paps./mouse = numbers of papillomas/mouse 24 weeks after beginning promotion.

[c] Carcin./mouse = number of carcinomas/mouse 52 weeks after beginning promotion.

COMPARISON OF THE TUMORIGENIC RESPONSE OF SENCAR AND C57BL/6 MICE TO BENZO(A)PYRENE AND THE INTER-EXPERIMENTAL VARIABILITY OVER A THREE-YEAR PERIOD

by

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Key words: SENCAR mouse, C57BL/6 mouse, benzo(a)pyrene, 12-O-tetradecanoylphorbol-13-acetate, inter-experimental variability, strain specificity, benzoyl peroxide.

SUMMARY

SENCAR and C57BL/6 mice were compared for their ability to produce tumors after benzo(a)pyrene [B(a)P] initiation and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) promotion. SENCAR mice initiated with 101 μg/mouse B(a)P and promoted with TPA (2 μg/mouse twice weekly) produced large numbers of papillomas while C57BL/6 mice produced none after 26 weeks of promotion. Continued treatment of the C57BL/6 mice with TPA up to 52 weeks did not induce any papillomas nor did higher doses of B(a)P. Application of increased doses of TPA (10 μg/ mouse, twice weekly) to B(a)P initiated C57BL/6 mice (404 μg/mouse) for 50 weeks produced few papillomas. Substantial papilloma formation in C57BL/6 mice was observed after weekly treatment with B(a)P (101 μg/mouse) with maximal production occurring at weeks 39-41 of treatment. In contrast, SENCAR mice treated according to the same protocol produced an equivalent response with maximal papilloma formation occurring 12-13 weeks earlier. Therefore, C57BL/6 mice exposed to B(a)P are capable of producing papillomas under certain experimental conditions.

The inter-experimental variability of B(a)P (50.5 μg/mouse) induced papilloma formation after 30 weeks of TPA promotion (2 μg/mouse, twice weekly) was examined in SENCAR mice over a 37 month period. Low statistical variation was observed in papilloma multiplicity, papilloma incidence, or papilloma latency. Male and female SENCAR mice produced equal values in the three parameters, 4.4 ± 1.6 papillomas/mouse, 87 ± 10% of the mice bearing papillomas and 9.6 ± 1.3 weeks to 10% of the mice bearing papillomas. The numbers of papillomas per mouse did not follow a Poisson distribution.

These data can serve as a reference source for future comparisons of mouse skin tumor initiation results between SENCAR and other strains or stocks of mice and with other chemicals.

INTRODUCTION

The SENCAR mouse was originally developed by Boutwell (1) by outbreeding Rockland STS mice with Charles River CD-1 mice against a selective pressure of 7,12-dimethylbenz(a)anthracene (DMBA) initiation and 12-O-tetradecanoylphorbol-13-acetate (TPA) promotion for 8 generations (2). Because of its demonstrated increased sensitivity to the tumor initiating effects of DMBA and benzo(a)pyrene [B(a)P] (3,4) this mouse has been the subject of intensive investigations using both complex mixtures and pure chemicals. We have reported in a series of publications the tumor initiating, tumor promoting, tumor co-initiating and complete carcinogenic properties of a wide variety of complex mixtures including gasoline exhausts, diesel exhausts, coke oven and roofing tar emissions and cigarette smoke condensate (5-8). In terms of pure chemicals many polycyclic aromatic hydrocarbons and their derivatives have been examined using the SENCAR mouse bioassay system (9-11). Also evaluated have been respiratory carcinogenic metal salts (8) as well as a series of known bladder, liver and lung carcinogens (12).

In our studies we had the opportunity to compare the tumor initiating and complete carcinogenic effects of B(a)P in both SENCAR and C57BL/6 mice. In previous papers we have reported that C57BL/6 mice are insensitive to two stage initiation-promotion with B(a)P and TPA when the TPA dose was 4 μg, twice weekly (13). In the studies reported here, we have reconfirmed this phenomenon and have examined the response of C57BL/6 mice initiated with B(a)P and promoted with an increased dose of TPA. Also examined in C57BL/6 mice is the papillomagenic response of B(a)P administered as a complete carcinogen.

We have also examined the inter-experimental variation of papilloma response in SENCAR mice over a 37 month period. The results of these experiments are presented and discussed and these results may provide a reference source for future comparisons with results from other SENCAR mice experiments or for comparisons of responses with other mouse strains or stocks.

MATERIALS AND METHODS

Seven- to nine-week-old SENCAR mice bred at Oak Ridge National Laboratory or C57BL/6 mice (Charles River Breeding Laboratory) were used. There were 80 animals (40 of each sex) per treatment group. Animals were house in plastic cages (10 per cage) under yellow light with hardwood chip bedding, fed Purina chow and water *ad libitum*, and maintained at 22-23°C with 10 changes of air per hour. All mice were shaved with surgical clippers two days before the initial treatment, and only those mice in the resting phase of the hair cycle were used.

Under the tumor initiation protocol, B(a)P (Aldrich Chemical Co., Milwaukee, WI) was applied as a single topical treatment in 0.2 mL spectral quality acetone. Beginning one week after treatment, 2.0 µg TPA (CCR, Eden Prairie, MN) in 0.2 mL acetone was administered topically twice weekly unless otherwise specified. Under the complete carcinogenesis protocol, B(a)P was administered in 0.2 mL acetone weekly for 52 weeks.

Skin tumor formation was recorded weekly, and papillomas greater than 2 mm in diameter and carcinomas were included in the cumulative total if they persisted for one week or longer. The number of mice with tumors, the number of mice surviving, and the total number of tumors were determined and recorded weekly. At six months, the number of papillomas per surviving animal was recorded for statistical purposes. The tumors were histologically verified.

RESULTS AND DISCUSSION

Comparison of SENCAR and C57BL/6 Mice

Using tumor initiation as the experimental protocol, mice were initiated with B(a)P over a wide dose range: SENCAR (0-101 μg/mouse); C57BL/6 (0-404 μg/mouse). Survival of both strains/stocks was very high at week 26 with greater than 95% of the mice surviving. Acetone initiated/TPA promoted control mice (males and females) gave the following response: SENCAR, 0.07 papillomas/mouse, 7% of mice bearing papillomas; C57BL/6, 0 papillomas/ mouse, 0% of mice bearing papillomas.

B(a)P elicited a strong dose-related effect in the production of papillomas in SENCAR mice (Fig. 1) while C57BL/6 mice exhibited no papillomas at any dose tested at 26 weeks. SENCAR mice were extremely sensitive to B(a)P initiation/TPA promotion as 5.4 μg of B(a)P induced tumors in 50% of the mice. The C57BL/6 mice were subjected to additional treatment and were continously dosed with TPA (2 μg/twice weekly) to week 52. Again, these mice did not exhibit any papillomas (or carcinomas) at any dose or in the TPA control group. The minimal survival at 52 weeks for the C57BL/6 mice was 88%. These results reconfirm our earlier findings on the insensitivity of C57BL/6 mice to the tumor promoting effects of TPA (13).

In order to attempt to induce papillomas in C57BL/6 mice, groups of 40 males and 40 females were initiated with 0, 202, and 404 μg/mouse of B(a)P and promoted with 10 μg of TPA twice weekly for 50 weeks. This dose of TPA was 5 times that used in the previous experiment (Fig. 1) and 2.5 times the dose reported by Reiners *et al.* (13). Survival was high at both 26 and 50 weeks of scoring with a minimum survival of 88% (Table 1).

Acetone initiated/TPA promoted mice again did not produce any papillomas or carcinomas. At 26 weeks a few papillomas were recorded at 404 μg/mouse of B(a)P and this number did not increase at 50 weeks. The largest number of papillomas was observed in the 202 μg/mouse group at 50 weeks, 0.35 papillomas/mouse with 22% of the mice bearing papillomas. In previous studies we have demonstrated that C57BL/6 mice initiated with B(a)P are susceptible to the tumor promoting properties of benzoyl peroxide (13). We conclude from the studies reported here and from previous studies that C57BL/6 mice are resistant to the tumor promoting effects of TPA rather than the tumor initiating effects of B(a)P.

A comparison of papilloma formation in male and female SENCAR and C57BL/6 mice induced by B(a)P under a complete carcinogenesis protocol was performed (Fig. 2). Mice were treated weekly with 101 μg/mouse of B(a)P. Papillomas were induced to about the same extent in both strains/stocks in multiplicity (~ 2 papillomas/mouse) and incidence (~ 90% of the mice bearing papillomas). However, the temporal production of tumors was dramatically different. Maximal papilloma formation in SENCAR mice occurred at 27-28 weeks while in C57BL/6 mice this occurred at 39-41 weeks, a shift of 12-13 weeks.

In previously reported studies, SENCAR and C57BL/6 mice were topically treated weekly with 12.5, 25.2, and 50.5 μg of B(a)P. Under these experimental conditions, C57BL/6 mice produced few papillomas at any time point while SENCAR mice produced approximately 1 papilloma/mouse with 40% of mice bearing papillomas between weeks 30-40 of treatment at the highest dose (13). The studies reported here extend and augment the published investigations using a 2-fold higher dose of B(a)P. Papilloma formation in C57BL/6 mice is clearly evident at the higher dose of B(a)P and is equivalent in both

SENCAR and C37BL/6 mice. The reasons for the temporal shift in papilloma formation between SENCAR and C57BL/6 mice are unknown. Several explanations are possible including increased resistance of C57BL/6 mice to B(a)P induced papillomagenesis or increased cytotoxic effects of B(a)P on C57BL/6 mouse epidermis with subsequent production of populations of cells resistant to the cytotoxic effects of B(a)P.

Since both SENCAR and C57BL/6 mice produce large numbers of carcinomas after weekly administration of B(a)P (12.5-50.5 µg/mouse/week) these results suggest that production of carcinomas and papillomas may not be directly related in C57BL/6 mice as suggested for other mouse strains (14).

Inter-experimental Variability of B(a)P Induced Papillomas

A series of 25 experiments with male and female SENCAR mice treated with B(a)P, 50.5 µg/mouse, and promoted with TPA (2 µg, twice weekly) were begun over a 37 month period between April 1979 and May 1982. In these experiments the same suppliers of B(a)P, TPA, and acetone were used as well as the same barrier conditions and breeding protocols for producing SENCAR mice.

Individual mice were scored at 30 weeks and the results of these studies presented in Figures 3-5. The mean responses (± S.D.) and ranges for papilloma incidence for male and female mice were 87 ± 10% (65-100%) and 86 ± 10% (65-100%) respectively (Figs. 3,4). The coefficient of variation was the same for both sexes, 12%. Papilloma multiplicity for male and female mice was also equal, 4.4 ± 1.6 papillomas/mouse. The coefficient of variation was 36%. The ranges of papilloma multiplicity for male and female mice were: 1.6-8.4 and 1.7-7.7 respectively. In terms of median papilloma incidence scores, male mice exhibited 89% while females exhibited

87% mice bearing papillomas. Median papilloma multiplicity scores were 4.6 and 4.1 papillomas/mouse for male and female mice respectively.

Skin papilloma latency was determined by measuring the time at which 10% of the mice bore papillomas (Fig. 4). The mear, S.D., range and median values in weeks for male mice were: 9.4, 1.1, 7-12, 10; for female mice: 9.8, 1.4, 7-13, 10. Based on the three parameters; papilloma incidence, papilloma multiplicity, and papilloma latency, equivalent responses and variations were observed for male and female SENCAR mice under these experimental conditions.

Except for a few sporadic points there seems to be no consistent time related effects with the production of mouse skin papillomas in SENCAR mice over a 3 year period. The inter-experimental variation is relatively small by any of the three parameters measured and probably reflects the consistent breeding scheme used in the production of SENCAR mice.

Skin papilloma distribution in B(a)P initiated TPA promoted male and female SENCAR mice was determined from 15 experiments at 30-32 weeks of promotion (Fig. 5). A non-Poisson distribution was observed with many mice bearing 0 to 6 papillomas/mouse and sporatic occurrences of mice with greater than 12 papillomas/mouse. One mouse had 26 papillomas. Drinkwater and Klotz (15) have reported that mouse skin papilloma multiplicity data follows a negative binomial distribution. It is evident from these studies that statistical comparisons using normal or Poisson assumptions cannot be applied to this data. In this regard we have reported on the use of a log probit analysis with non-zero background (16) which can be used to analyze mouse skin papilloma data (5,6).

These data provide a useful tool for future comparisons of mouse skin tumor initiation data in SENCAR or other strains or stocks of mice with B(a)P or with other chemicals and can serve as a reference source for this information.

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Table 1. Mouse skin tumor initiation in C57BL/6 mice by B(a)P using higher doses of TPA[a]

Dose, µg/Mouse	Number of Mice Surviving[b]		Mice Bearing Papillomas[b], %		Papillomas/ Mouse[b]	
	M	F	M	F	M	F
0	40 (39)	39 (38)	0 (0)	0 (0)	0 (0)	0 (0)
202	39 (37)	38 (35)	3 (22)	5 (11)	0.07 (0.35)	0.05 (0.11)
404	38 (36)	40 (37)	10 (6)	10 (14)	0.10 (0.05)	0.10 (0.13)

[a] Mice promoted with TPA 10 µg, twice weekly, one week after initiation.

[b] Mice scored 26 weeks and 50 weeks of promotion. Values in parenthesis are data after 50 weeks of promotion.

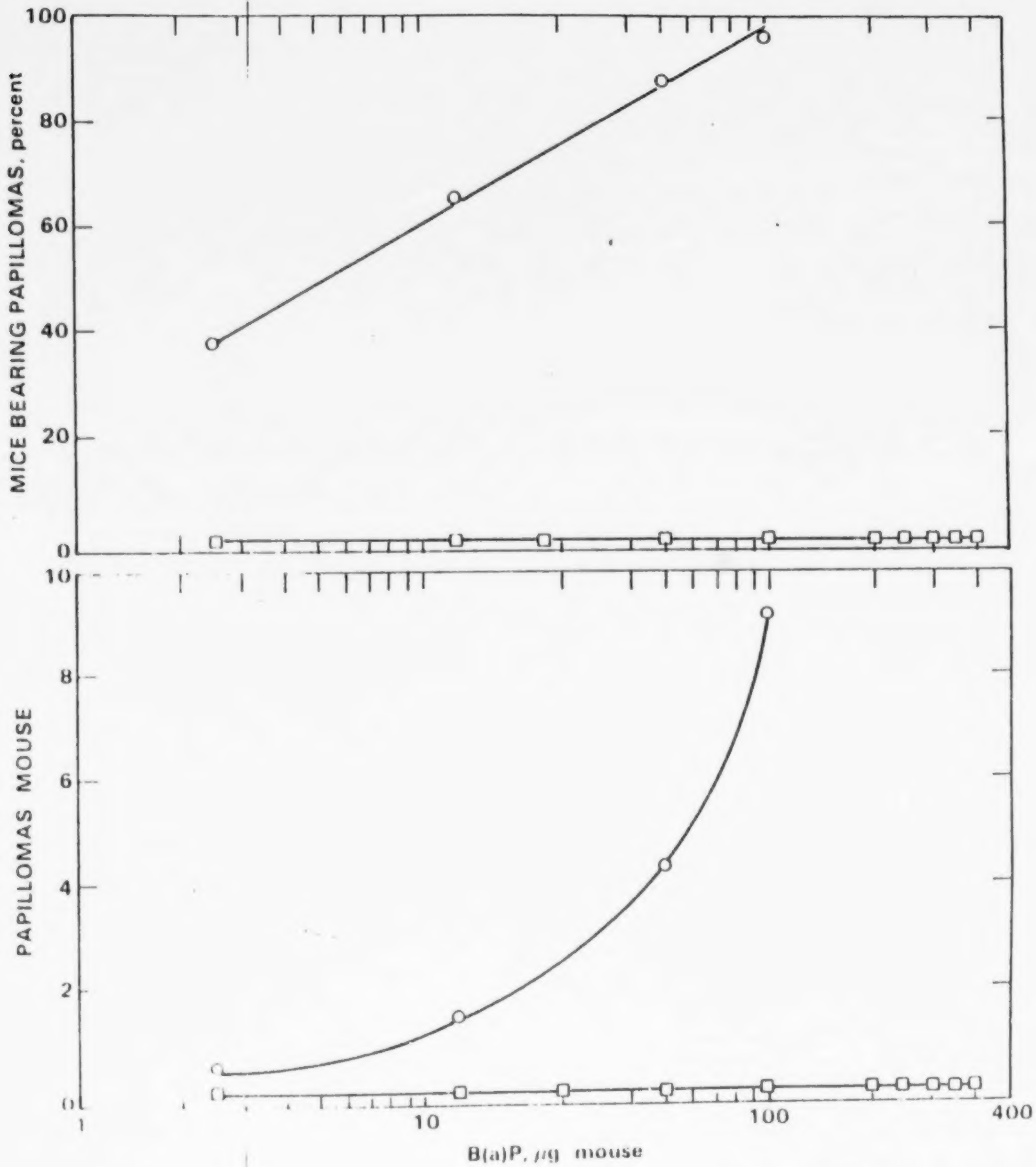

Figure 1. Comparison of mouse skin tumor initiation induced by B(a)P in male and female SENCAR and C57BL/6 mice. Mice were initiated with B(a)P and promoted twice weekly with 2 µg of TPA.

● SENCAR, ○ C57BL/6

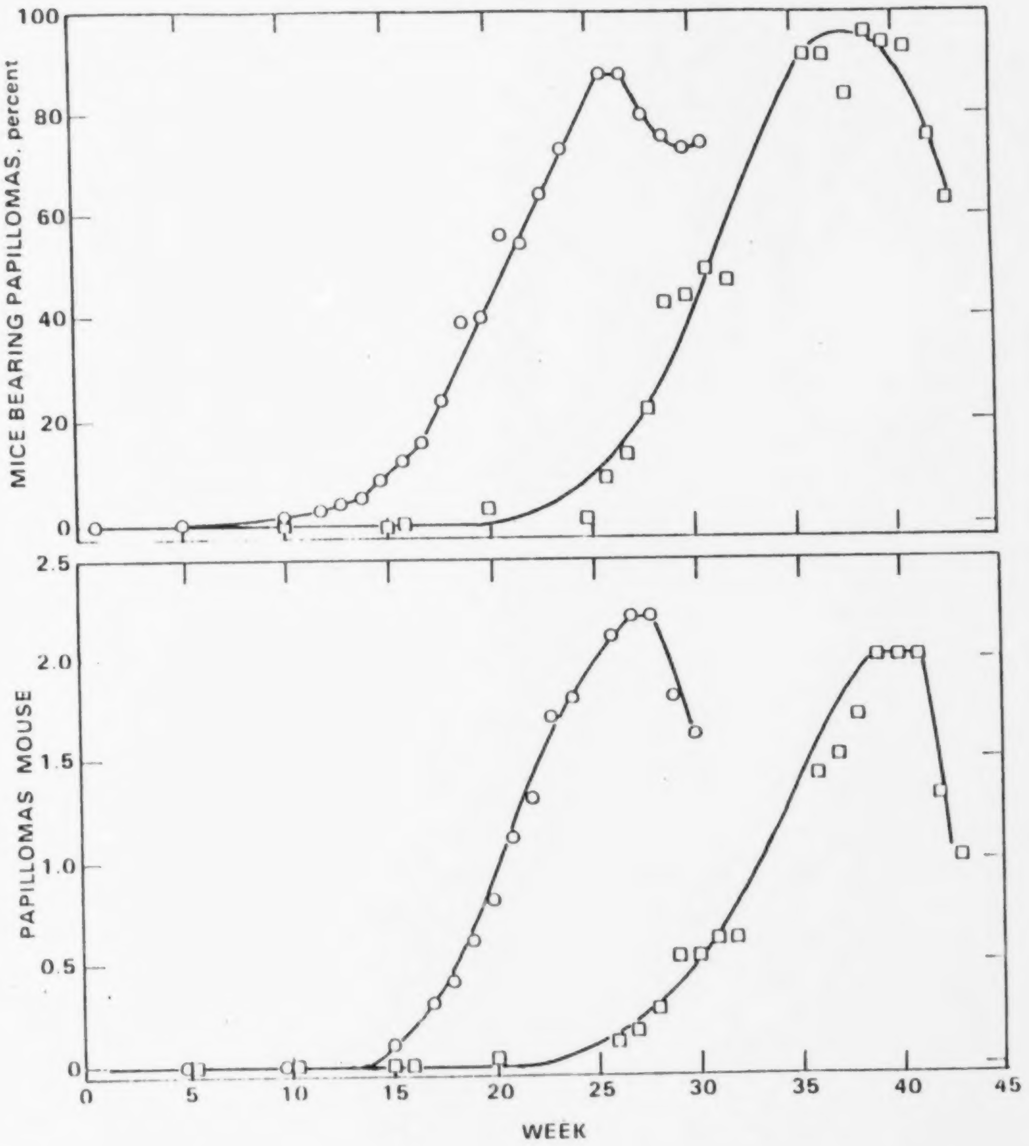

Figure 2. Comparison of mouse skin complete carcinogenesis induced by B(a)P in male and female SENCAR and C57BL/6 mice. ● SENCAR, ○ C57BL/6

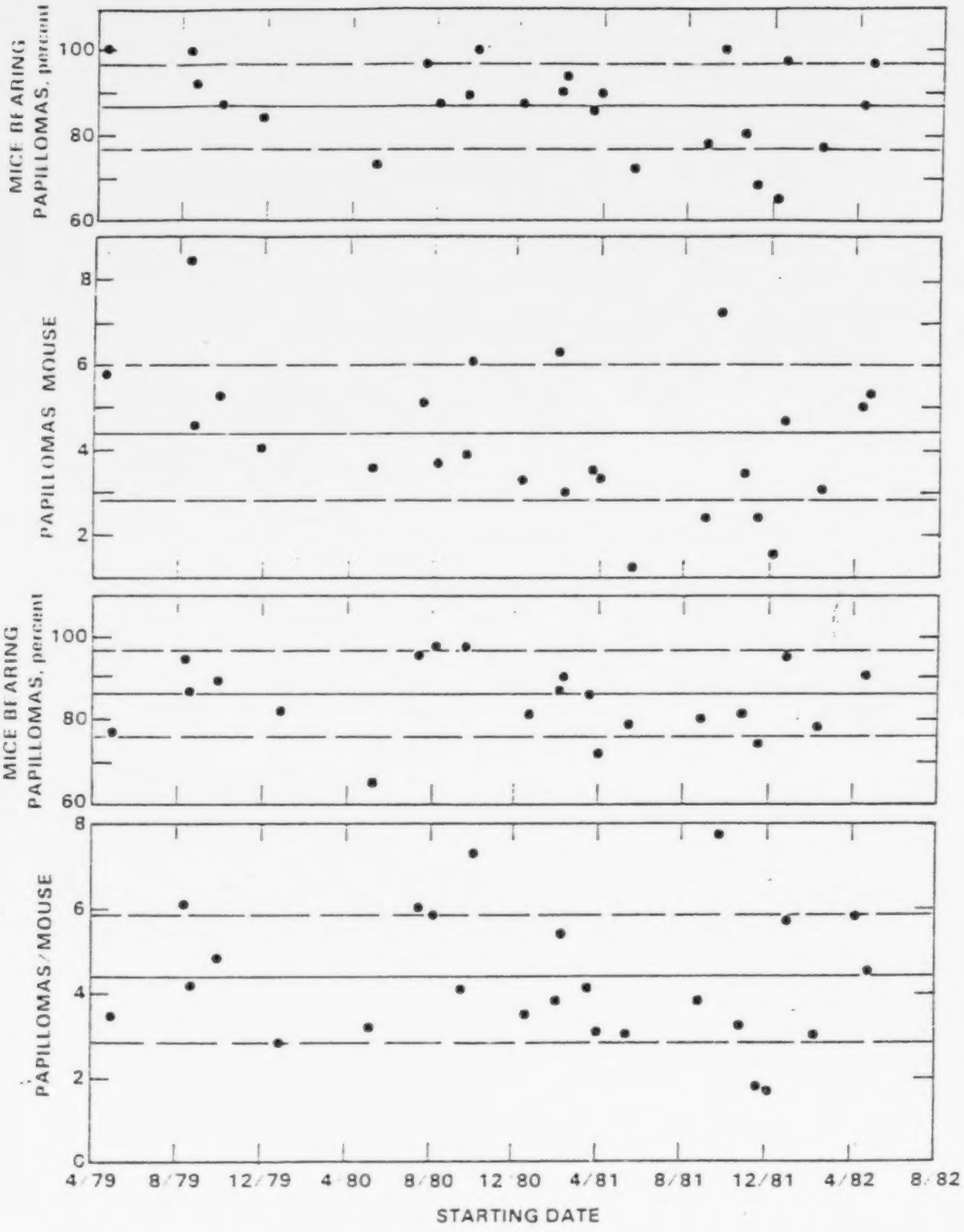

Figure 3. Inter-experimental variation of skin papilloma incidence and multiplicity in B(a)P initiated/TPA promoted SENCAR mice. Male and female mice were initiated with B(a)P, 50.5 μg/mouse and subsequently treated one week later with TPA (2 μg, twice weekly) for 30 weeks. Each data point represents an initial group of 40 mice. Average survival was: male, 95%, female, 93%. Upper panel, male mice; lower panel, female mice. Shaded areas are ± 1 S.D.

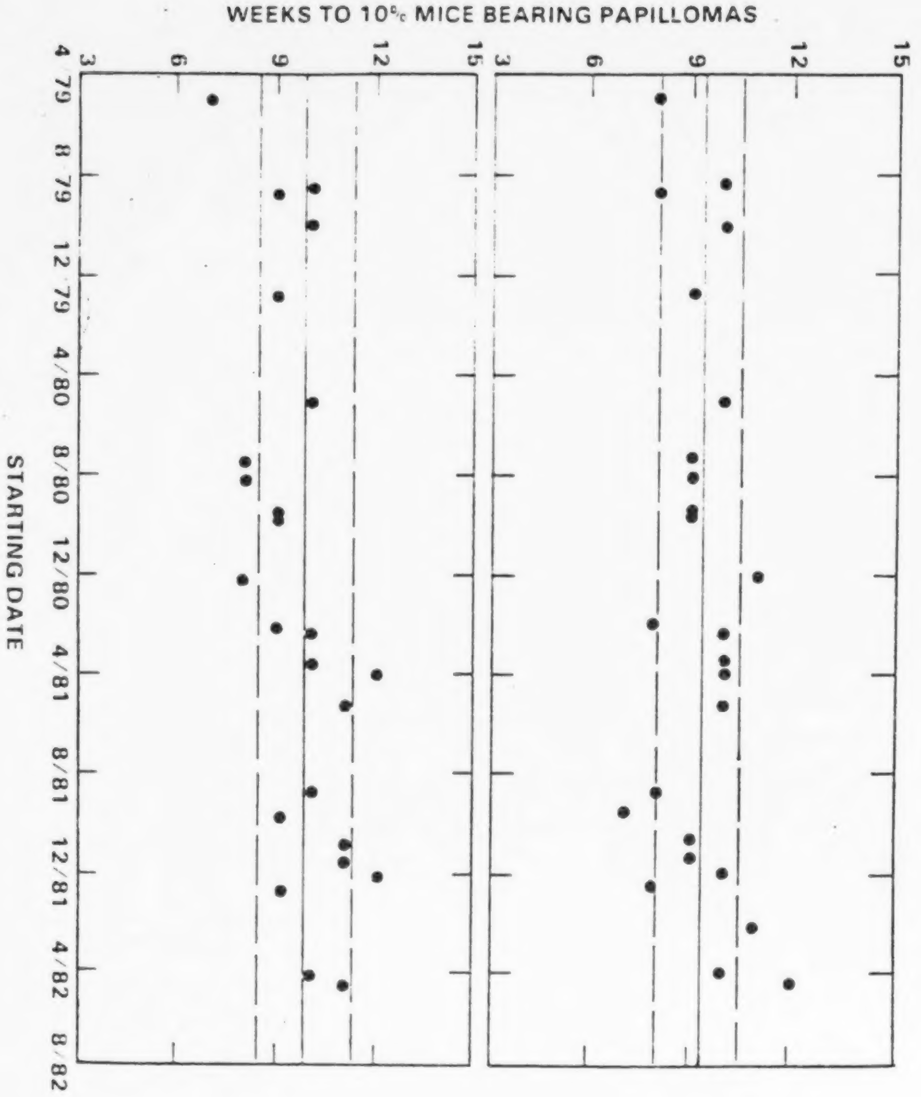

Figure 4. Inter-experimental variation in skin papilloma latency in B(a)P initiated/TPA promoted SENCAR mice. See Figure Legend 2 for experimental details. Upper panel, male mice; lower panel, female mice. Shaded areas are ± 1 S.D.

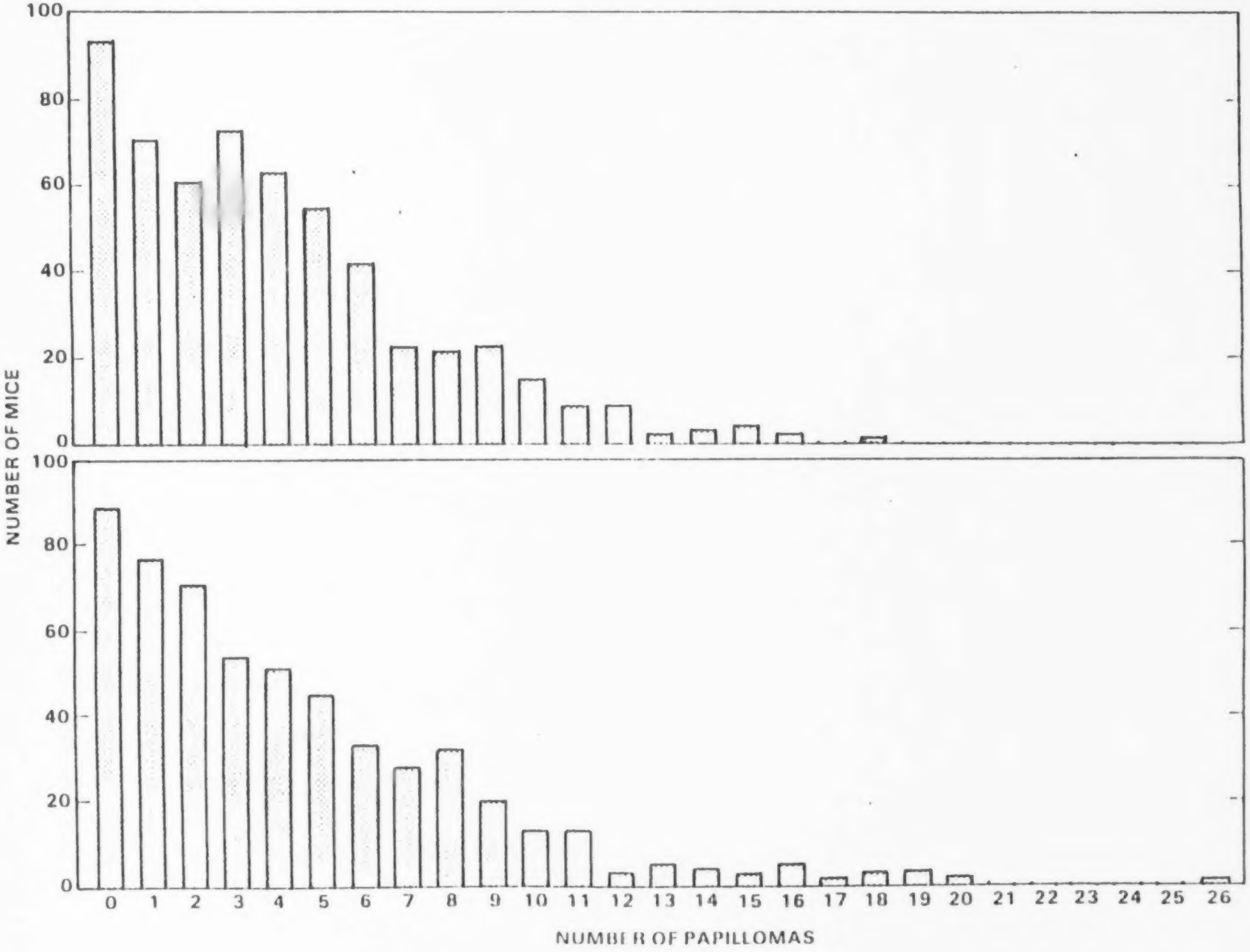

Figure 5. Distribution of skin papillomas in B(a)P initiated/TPA promoted SENCAR mice. Results presented are combined from 15 experiments with male or female mice in which individual animals were scored at week 30-32. See Figure Legend 2 for experimental details. Upper panel, male mice; lower panel, female mice.

SENCAR MOUSE SKIN TUMORIGENESIS MODEL VERSUS OTHER STRAINS AND STOCK OF MICE

by

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Key Words

SENCAR mouse

Skin carcinogenesis

Tumor initiation

Tumor promotion

Papillomas

Carcinomas

DMBA

MNNG

TPA

Benzoyl peroxide

Abstract

The SENCAR stock of mice was selectively bred for sensitivity to skin tumor induction by the two-stage tumorigenesis protocol for eight generations using 7,12-dimethylbenz(a)anthracene (DMBA) as the initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as the promoter. The SENCAR mouse was derived from crossing Charles River CD-1 mice with skin tumor sensitive mice (STS). The SENCAR mice are much more sensitive to both DMBA tumor initiation and TPA tumor promotion than CD-1, BALB/c, and DBA/2 mice. There is an even greater difference in the sensitivity to two-stage skin tumorigenesis between SENCAR and C57BL/6 mice when using DMBA-TPA treatment. However, the SENCAR and C57BL/6 mice have a similar tumor response to DMBA-benzoyl peroxide treatment suggesting that TPA is not an effective promoter in C57BL/6 mice. The DBA/2 mice respond in a similar manner to the SENCAR mice when using N-methyl-N-nitro-N-nitrosoquandine-TPA treatment. There is a good dose-response relationship of many carcinogens used as tumor initiators and of many compounds used as tumor promoters in the SENCAR mouse. When compared to other stocks and strains of mice, the SENCAR mouse has one of the largest data bases of carcinogens and promoters.

Introduction

It is well known that skin tumors (papillomas, keratoacanthomas, and squamous cell carcinomas) can be induced in mice by a complete carcinogenesis protocol (carcinogen given repetitively) or by the sequential application of a subthreshold dose of a carcinogen (initiation stage) followed by repetitive treatment with a weak or non-carcinogenic tumor promoter (promotion stage). The initiation stage requires only a single application of either a direct or an indirect carcinogen at a subthreshold dose and is essentially irreversible, while the promotion stage is brought about by repetitive treatments after initiation and is reversible for a period of time but later becomes irreversible (1,2).

In order to better understand the SENCAR mouse and its relative sensitivity to skin carcinogenesis when compared to other stocks and strains of mice, it is necessary to first discuss the derivation of the SENCAR mouse.

Derivation

The SENCAR stock of mice was selectively bred for sensitivity to skin tumor induction by two-stage tumorigenesis. Consequently, the SENCAR mouse is extremely sensitive to two-stage carcinogenesis and coincidentally sensitive to complete carcinogenesis. The SENCAR mouse was derived from crossing Charles River CD-1 mice with skin tumor sensitive mice (STS) and selecting for sensitivity to 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) two-stage carcinogenesis for 8 generations starting with the F_1 cross as originally described by Boutwell (2). Figure 1 outlines the method used by Boutwell to select for the STS mice. A similar selection procedure was used by Boutwell and coworkers to derive the SENCAR mice as outlined in Figure 2. In both cases, the mice developing the first and most papillomas after initiation-promotion treatment were selected for each breeding.

Comparison to Other Stocks and Strains of Mice. The SENCAR mice are between 10 and 20 times more sensitive to DMBA tumor initiation than the CD-1 mice (Table 1). However, as shown in Table 2, they are only between 3 and 5 times more sensitive to benzo(a)pyrene (B(a)P) tumor initiation than the CD-1 mice (3). In addition, the SENCAR mice are 2 to 3 times more sensitive to TPA promotion than the CD-1 mice (3). In addition, BALB/c mice appear to be consistently less sensitive to two-stage tumorigenesis than the SENCAR mice (4).

Although there exist several stocks and strains of mice that have been used in skin tumor induction experiments, very little dose-response data is available as well as very few comparative studies on the relative sensitivity except for SENCAR and C57BL/6 mice. There is an even greater difference in the sensitivity to two-stage skin carcinogenesis between SENCAR and C57BL/6 mice than between the SENCAR and CD-1 mice. C57BL/6 mice are very refractory to two-stage skin carcinogenesis by B(a)P-TPA. As shown in Table 3, even high initiating doses of

B(a)P (1600 nmoles) and high promoting doses of TPA (10 g) are quite ineffective in causing skin tumors in C57BL/6 mice. However, C57BL/6 mice do respond to complete carcinogenesis by B(a)P and, in this regard, they appear to be equal to or slightly more sensitive than the SENCAR mice (5). This unequal susceptibility to complete and two-stage carcinogenesis within a stock or strain of mice strongly suggests that the promotional phases of complete and two-stage carcinogenesis are dissimilar. In addition, differences in sensitivity to initiation and promotion between mice may be due to alterations in the promotional phase of two-stage carcinogenesis. In this regard, we have recently found that benzoyl peroxide is an effective promoter in C57BL/6 and SENCAR mice (5). For some reason, TPA is not an effective promoter in C57BL/6 mice.

Recently, DiGiovanni and coworkers (6) have found that the DBA/2 mice are much less sensitive to DMBA-TPA two-stage tumorigenesis than the SENCAR mice. If high initiating doses (400 nmoles/mouse) of DMBA are used on the DBA/2 mice followed by TPA promotion, they do respond with a high tumor yield. When DBA/2 mice are compared with SENCAR mice following initiation with N-methyl-N^{1}-nitro-N-nitrosoquandine (MNNG) and TPA promotion, the DBA/2 and SENCAR mice respond with a nearly identical tumor response (6).

Carcinogens and/or Initiators Used in SENCAR Mice. The SENCAR mouse model has presently one of the largest data bases of carcinogens and/or initiators. All the carcinogens and/or initiators that have been tested in both the CD-1 and SENCAR mice have been found to be positive in both stocks of mice. The major difference is related to the increased sensitivity and shorter latency period to tumor development in the SENCAR mice. As was the case in CD-1 mice, DMBA and B(a)P show a good dose-response relationship in SENCAR mice (Table 4). As can be seen a good correlation exists between the number of papillomas per mouse at 15 weeks and the final carcinoma incidence at 50 weeks. The percent of mice with papillomas also has a reasonable correlation but the dose-response is very narrow (Table 4).

Many carcinogenic polycyclic aromatic hydrocarbons (PAH), metabolites, and derivatives have been determined in the SENCAR mouse skin tumorigenesis model (4,7-27). Besides the PAH's, a number of other classes of chemical carcinogens have been found to be positive in a dose-response relationship as skin carcinogens and/or initiators in the SENCAR mouse such as sterigmatocystin, urethane, 1,3-propanesultone, 4-nitroquinoline-N-oxide, MNNG, N-nitrosoethylurea (ENU), N-nitrosomethylurea, epicholorohydrin, and β-naphthylamine (Slaga and coworkers, unpublished data). In this regard both MNNG and ENU have been found to be very potent skin carcinogens and tumor initiators which suggest that these compounds will be useful direct acting carcinogens in the skin model (Slaga and Nesnow, unpublished data). Several other chemicals (dimethylnitrosamine, dimethylhydrazine, acetylaminofluorine and 4-amino-biphenyl) which are positive as carcinogens in certain tissues have been extensively studied in SENCAR mouse skin and found to be negative (Slaga and coworkers, unpublished data). A possible explanation may be the lack of metabolic activation of these compounds in the skin. In addition, several controversial compounds such as chloroform and malonaldehyde were also found to be negative in the SENCAR mouse skin tumorigenesis model (Slaga and coworkers, unpublished data).

A number of environmentally important complex mixtures have also been extensively studied for skin carcinogenic and/or tumor initiating activity in the SENCAR mouse (28-32). Of the complex mixtures tested, coke oven main and roofing tar had both strong skin carcinogenic and tumor initiating activity, whereas the Nissan diesel exhaust sample was only a strong tumor initiator.

Promoters Used in SENCAR Mice. Also the case with carcinogens and/or tumor initiators, not all of the various skin tumor promoters tested have been assayed in SENCAR mice. As shown in Table 5, a good dose-response relationship exists for TPA skin tumor promotion after DMBA initiation when considering either the number of papillomas per mouse at 15 weeks or the percent of mice with squamous cell carcinomas at 50 weeks. The repetitive application of the promoter TPA without initiation by DMBA in general gives rise to a few tumors but a dose-response relationship has never been noted (Table 6). The maximum response observed after 50 weeks of treatment with TPA was a 22% incidence of mice with papillomas and 5% with carcinomas.

Although the initiation stage appears to be irreversible, the promotion stage is reversible requiring a certain frequency of applications in order to induce tumors. Table 7 compares the promoting activity of various doses of TPA when given either three times, two times or one time per week. In general, as the frequency of application of TPA decreases the promoting activity also decreases. It should be pointed out, however, that one application per week of TPA after initiation by 20 nmoles of DMBA is still fairly effective in tumor promotion. Even high doses of TPA given once every two weeks or once every three weeks are ineffective in tumor promotion (33).

Besides TPA, several other phorbol ester tumor promoters have been assayed in SENCAR mice. Phorbol 12,13-dibutyrate and 12-deoxyphorbol-13-decanoate both show a good dose-response relationship (Slaga and coworkers unpublished data). Although 4-O-methyl-TPA is generally not considered a tumor promoter, it does have some promoting activity at doses greater than 200 μg per application. 12-O-Retinylphorbol-13-acetate was also found to have skin tumor promoting activity but less than TPA. In general, mezerein can be considered a weak skin tumor promoter in SENCAR mice (33,34).

A number of other chemicals have been found to have skin tumor promoting activity in SENCAR mice such as teleocidin, retinoic acid, benzoyl peroxide, lauryl peroxide, cumene peroxide, decanyl peroxide, cumene hydroperoxide, terylbutyl-hydroperoxide, butylated hydroxytoluene-hydroperoxide, 1-fluoro-2, 4-dinitrobenzene, benzo(e)pyrene, anthralin and chrysarobin (1,33,35 and Slaga and coworkers, unpublished data). A number of chemicals have been found to be negative or very weak as skin tumor promoters in SENCAR mice such as hydrogen peroxide, hydroquinone, butylated hydroxyanisole, butylated hydroxytoluene, calcium ionophore A23187, chloroquine, Quabain, chlorpromazine, epichlorohydrin, chloroform, malonaldehyde, phenanthrene, 2-amino-anthracene, 1-amino-anthracene, phenanthrenequinone, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Slaga and coworkers, unpublished data).

In terms of complex mixtures, diesel fuel was found to have a moderate skin tumor promoting activity but negative as a carcinogen in SENCAR mice (Slaga and coworkers, unpublished data).

Conclusion

The SENCAR mouse is, in general, one of the most sensitive stocks and strains of mice presently available for both complete and two-stage carcinogenesis studies. The SENCAR mouse skin tumorigenesis model has one of the largest data bases for skin carcinogens, tumor initiators, tumor promoters, and anticarcinogens (anti-initiators and anti-promoters). It gives excellent dose-responses with both pure substances and complex mixtures. The two-stage tumorigenesis protocol can be considered a reliable and relatively short-term bioassay for carcinogens and promoters. This model has also been very important in determining the mechanism of action of carcinogens, promoters, and anticarcinogens.

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Table 1 Comparison of the tumor-initiating activity of DMBA in SENCAR *vs* CD-1 mice [a]

Each experimental group is an average of three experiments containing 30 mice per experiment. DMBA was applied topically once and followed 7 days later by twice weekly promoting applications of 8.5 nmol of TPA[b]

Mice	DMBA (Dose, nmol)	# of papillomas per mouse at 15 wks.	# of mice with papillomas at 15 wks	# of papillomas per mouse at 25 wks	% of mice with papillomas at 25 wks
SENCAR	100	22.0	100	24.0	100
SENCAR	10	6.4	100	7.0	100
SENCAR	1	3.2	90	3.8	95
SENCAR	0.1	0.3	15	0.6	20
SENCAR	0.0[c]	0	0	0.1	10
CD-1	100	4.8	75	5.6	90
CD-1	10	2.2	60	3.0	72
CD-1	1	0	0	0.2	10
CD-1	0.1	0	0	0	0
CD-1	0.0[c]	0	0	0.2	10

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[a] Data taken from Reference 33

[b] The maximum percent standard deviation for all groups was 15%.

[c] TPA only group; the mice received twice weekly applications of 8.5 nmol of TPA for 25 wks.

Table 2 Comparison of the tumor-initiating activity of BP in SENCAR *vs.* CD-1 mice[a]

Mice	DMBA (Dose, nmol)	# of papillomas per mouse at 15 wks.	# of mice with papillomas at 15 wks	# of papillomas per mouse at 25 wks	% of mice with papillomas at 25 wks
SENCAR	200	7.6	100	8.2	100
SENCAR	100	3.4	78	3.8	80
SENCAR	50	1.4	56	1.6	60
SENCAR	10	0.6	36	0.9	42
SENCAR	0[c]	0	0	0.1	10
CD-1	200	1.4	56	3.8	72
CD-1	100	1.0	44	1.8	58
CD-1	50	0.5	36	0.7	40
CD-1	10	0	0	0.1	10
CD-1	0[c]	0	0	0.1	10

[a] Data taken from Reference 33

[b] The maximum percent standard deviation for all groups was 18%

[c] TPA only groups; the mice received twice weekly applications of 8.5 nmol of TPA for 25 weeks.

Table 3 Initiation-promotion in SENCAR and C57BL/6 mice[a]

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1. Repetitive applications of various dose levels of TPA for 52 weeks without initiation in SENCAR mice will give a low level of papillomas (5-20%) and carcinomas (<15%) but does not show a dose response relationship. Benzoyl peroxide gives less than 5% tumors in similar experiments.

2. Repetitive applications of various dose levels of TPA or benzoyl peroxide after initiation in SENCAR mice will give a dose-response in terms of papillomas (early) and carcinomas (late).

3. Repetitive applications of various dose levels of TPA or benzoyl peroxide for 52 weeks without initiation in C57BL/6 mice did not give any tumors.

4. Repetitive applications of various dose levels of TPA after initiation in C57BL/6 mice gave a very low level of papillomas (<5%) and carcinomas (<10%). (A) In this experiment BP doses of 50 to 1600 nmoles were used to initiate the mice.

5. Repetitive applications of benzoyl peroxide after initiation in C57BL/6 mice gave a 45% carcinoma incidence.

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[a] See reference 5

Table 4 Dose response studies on the ability of DMBA and BP to initiate skin tumors in SENCAR mice[a]

The mice were treated one week after initiation with twice weekly applications of 5 ug of TPA

Initiator	Dose (nmoles)	# of papillomas per mouse at 15 weeks	% of mice with papillomas at 15 weeks	% of mice with carcinomas at 50 weeks
DMBA	100	22.0	100	100
DMBA	10	6.8	100	40
DMBA	1	3.2	93	22
DMBA	0.1	0.5	20	5
BP	200	7.5	100	55
BP	100	3.2	78	30
BP	50	1.4	60	18

[a] Data taken from Reference 33

Table 5 Dose-response studies on the ability of TPA to promote tumors after DMBA initiation [a]

The mice were initiated with 10 nmoles of DMBA and promoted one week later with various dose levels of TPA

Promoter	dose (ug)	Time to first papilloma (wks)	# of papillomas per mouse at 15 wks	with papillomas at 15 wks	with carcinomas at 50 weeks
TPA	10	8	3.0	100	32
TPA	5	6	7.2	100	46
TPA	2	7	6.5	100	45
TPA	1	8	3.6	80	25
TPA	0.1	11	0.4	5	8

[a] See Reference 33 for details

Table 6 Dose-response studies on the ability of TPA to act as a complete carcinogen in SENCAR mice[a]

TPA Dose (ug)	# of papillomas per mouse at 50 wks	% of mice with papillomas at 50 wks	% of mice with carcinomas at 50 wks
0	0	0	0
1	0.09	8	0
2	0.07	7	0
4	0.20	22	5
6	0.15	12	0

[a] The mice (80 per group) were treated twice weekly with various dose levels of TPA

Table 7 Comparison of the frequency of application and dose of TPA on skin tumor promotion[a]

DMBA Initiation (dose, nmoles)	TPA (dose, ug)	Frequency of Application	Papillomas per mouse at 16 wks	% of mice with papillomas at 16 wks
10	1	3x/wk	7.8	100
10	1	2x/wk	3.6	80
10	2	2x/wk	6.5	100
10	5	2x/wk	10.2	100
10	10	2x/wk	4.6	100
10	0.5	1x/wk	1.0	40
10	1	1x/wk	1.8	66
10	2	1x/wk	3.2	74
10	3	1x/wk	4.3	78
10	4	1x/wk	5.1	82
10	5	1x/wk	6.4	86
20	1	1x/wk	4.2	76
20	2	1x/wk	6.0	96
20	4	1x/wk	9.0	100

[a] 30 female SENCAR mice were used for each group. Slaga _et_ _al_. unpublished results.

FIGURE 1

Outline of the Method Used in the Selective Breeding Experiments for Skin Tumor Sensitive (STS) and Resistant (STR) mice (Boutwell)

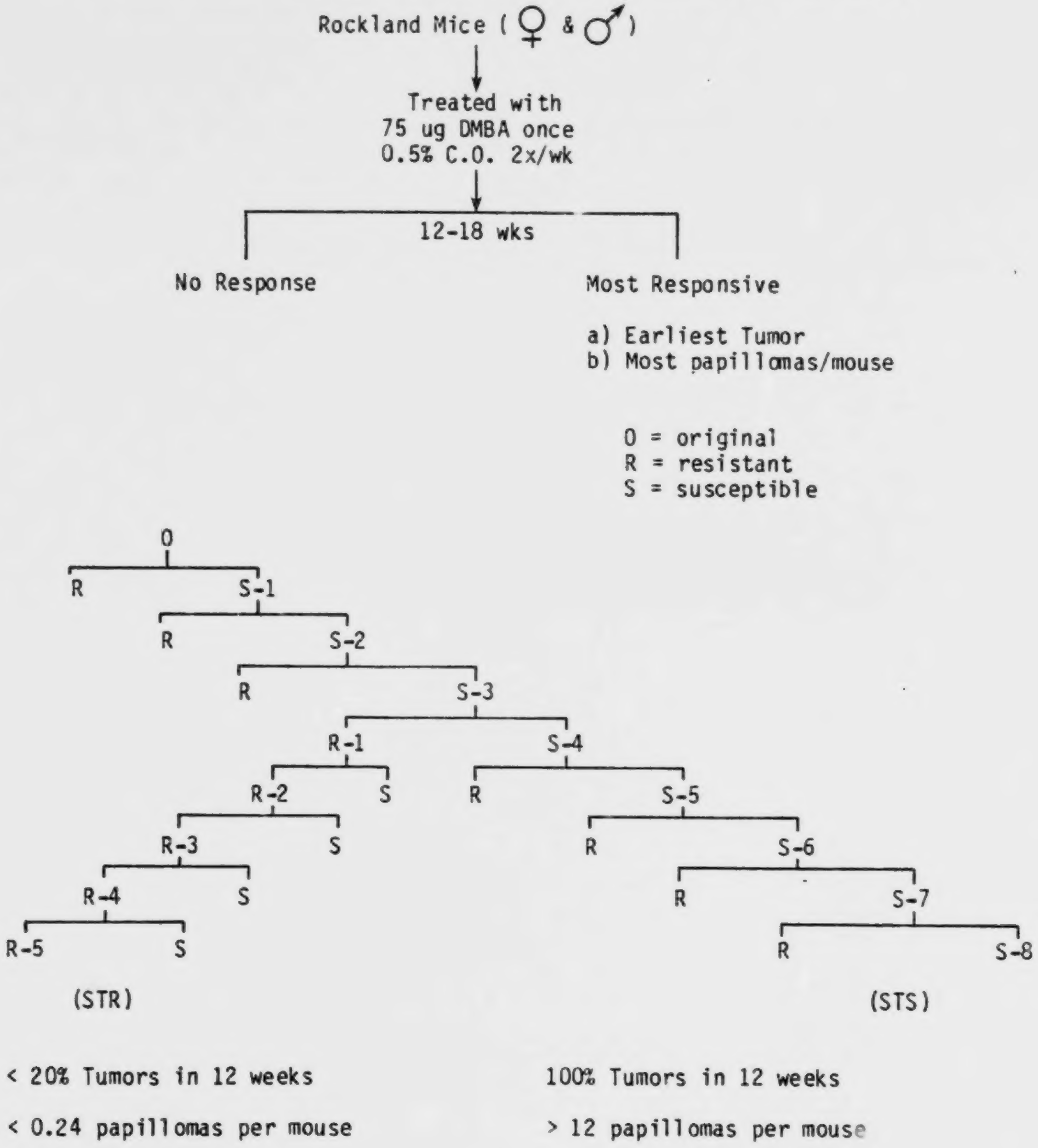
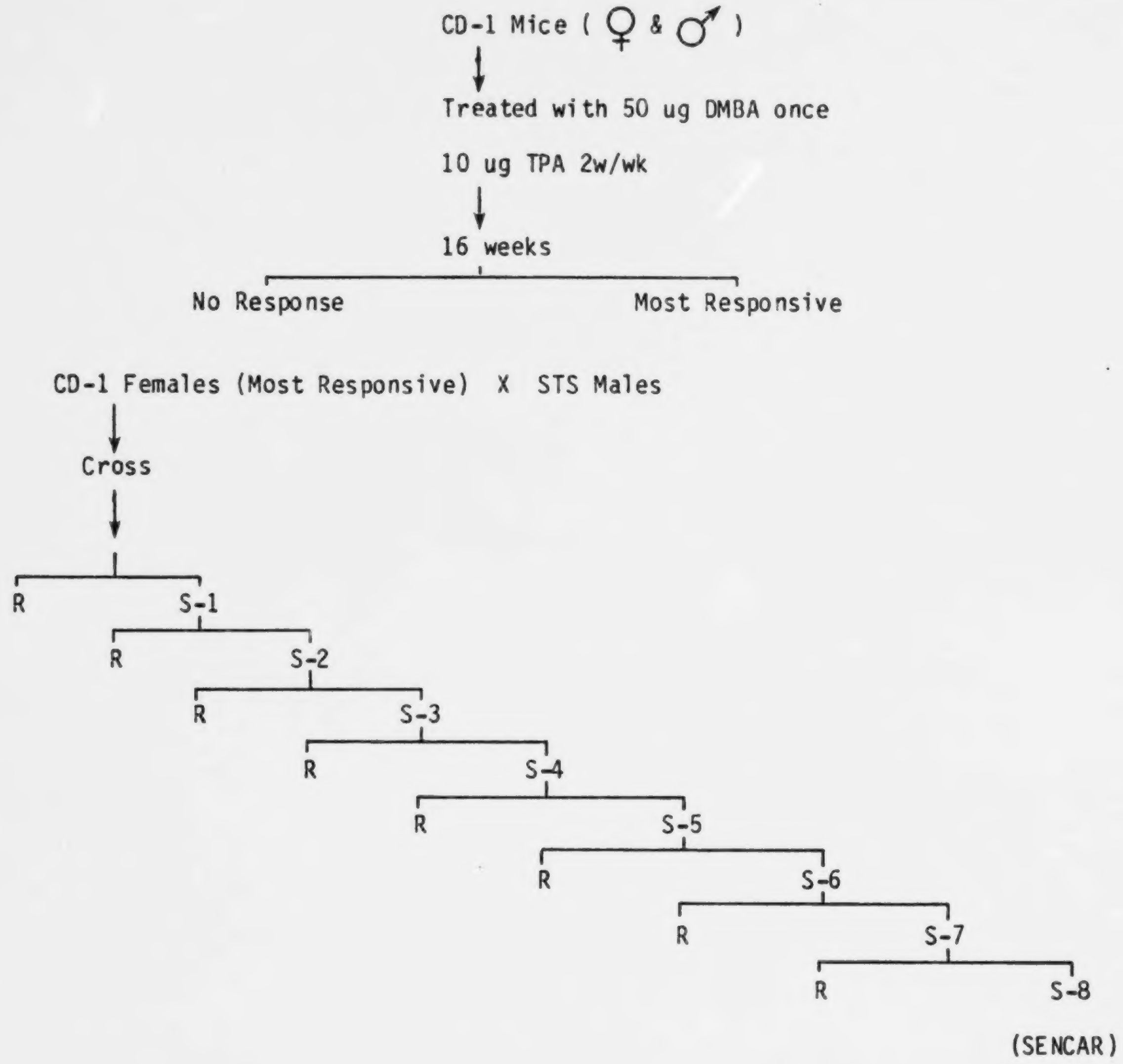


FIGURE 2

Outline of the Method Used in the Selective Breeding Experiments for SENCAR Mice (outbreeding)



IN VITRO COMPARISONS OF SENCAR AND BALB/c PRIMARY EPIDERMAL CELLS

by

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Key words: susceptibility, SENCAR, carcinogenesis, tumor promotion, EGF, TPA, DNA repair, Langerhans cell, retrovirus, Balb/c

ABSTRACT

Grafting experiments show that the enhanced sensitivity of the SENCAR mouse to skin carcinogenesis by initiation and promotion is a property of the skin itself, suggesting the usefulness of in vitro studies to elucidate the mechanism. Such studies have indicated that cultured epidermal cells of SENCAR and the resistant Balb/c strain are remarkably similar in a variety of respects. DNA repair and carcinogen binding are quantitatively similar in cultured cells of SENCAR and more resistant mouse strains. Epidermal Langerhans cell number and Langerhans cell-mediated functions were indistinguishable in SENCAR and Balb/c mice. Primary epidermal cells cultured in the presence of various concentrations of 12-O-tetradecanoylphorbol-13-acetate (TPA), retinoic acid, epidermal growth factor (EGF), hydrocortisone, or fluocinolone acetonide failed to reveal differences in growth between Balb/c and SENCAR cells. Cells from these animals bound comparable amounts of EGF with similar kinetics, and the modulation of this binding by TPA and retinoic acid was indistinguishable between strains. Spontaneous expression of infectious, endogenous xenotropic type C RNA virus at very low levels could be demonstrated in primary Balb/c epidermal cells and both Balb/c and SENCAR epidermal lines resistant to Ca^{2+}-induced terminal differentiation. The number of foci of initiated cells after exposure to carcinogens in vivo or in vitro did not differ significantly between SENCAR and Balb/c, suggesting that SENCAR sensitivity is primarily to promotion. However, there are qualitative differences between SENCAR and Balb/c foci. The appearance of foci of cells resistant to terminal differentiation in untreated SENCAR cultures supports the evidence from in vivo studies for the existence of a constitutively initiated cell population in SENCAR mouse skin.

INTRODUCTION

The SENCAR mouse was derived by selective breeding for rapid development of large numbers of papillomas after initiation with dimethylbenz[a]-anthracene (DMBA) and promotion with 12-O-tetradecanoylphorbol 13-acetate (TPA) (1). Subsequent studies have shown that the enhanced sensitivity exists for initiators with a variety of different chemical structures, including the directly acting N-methyl-N'-nitro-N'-nitrosoguanidine (MNNG), a carcinogenic nitrosamide which does not require metabolic activation (2). Such data, along with a report that metabolites of DMBA are similar in skin of SENCAR mice and the less sensitive parent CD-1 strain (1), strongly suggest that the enhanced sensitivity of SENCAR mice does not result from differences in metabolism of polycyclic aromatic hydrocarbon carcinogens. Further evidence tending to rule out metabolic differences came from a study that showed the SENCAR mouse to be more highly sensitive to skin carcinogenesis by a single dose of ultraviolet (UV) radiation than the more resistant CD-1 strain (3). A major finding in elucidating the mechanism of SENCAR sensitivity was that the susceptibility is a property of the skin itself rather than a systemically-mediated phenomenon (4). Skin of SENCAR and the resistant Balb/c mouse strain grafted to athymic nude mouse recipients retained the tumor incidence of the donor animals after initiation and promotion. This result validates the use of *in vitro* studies with cultured skin cells to elucidate the mechanism of SENCAR sensitivity and led to the work described in this paper. However, *in vivo* experiments have continued to be useful to define areas to be explored in culture and to confirm *in vitro* results. We have most frequently used Balb/c as our representative resistant strain for comparisons with the susceptible SENCAR mice since the work of Hennings *et al.* (2), using initiation-promotion *in vivo*, helped to define the basic biology of comparative epidermal carcinogenesis in these strains. In SENCAR mice more papillomas and carcinomas developed with a shorter latency period than in Balb/c animals. This was true whether adult or newborn mice were used and whether DMBA or MNNG were used as initiator, though DMBA was the stronger initiator. This study provided two additional pieces of information of possible significance: 1) SENCAR skin was notably more sensitive to toxic effects of TPA such that ulcerative lesions developed, requiring less frequent applications of promoter; 2) SENCAR mice, but not Balb/c, developed significant numbers of papillomas and carcinomas with TPA alone, in the absence of an exogenous initiator. This result may imply that SENCAR mouse skin contains a population of constitutively initiated cells. There is evidence from *in vitro* experiments, to be discussed later, which support such a hypothesis.

MATERIALS AND METHODS

Animals. SENCAR mice were obtained from the NCI Frederick Cancer Research Facility, Frederick, MD. Balb/c and other mouse strains were obtained through the NIH Small Animal Section, Bethesda, MD.

Cell culture. Primary epidermal cells were obtained from newborn ($\leq$ 3 days) mice by the trypsin flotation method as described previously (5) and cultured in Eagle's minimal essential medium (MA Bioproducts, Walkersville, MD) with 8% Chelex-treated fetal calf serum (Reheis Chemical Co., Kanakakee, IL) with final Ca^{2+} concentration in the medium adjusted to 0.05 mM to select for basal cells as described by Hennings *et al.* (6). To induce terminal differentiation, the Ca^{2+} concentration was raised to 1.4 mM (6). Cell cultures were routinely incubated at 36.5° in a humidified atmosphere of 7% CO_2 in air. For cell counts, cells were removed from plastic culture dishes by incubation with 0.06% trypsin, 0.1% EDTA in phosphate-buffered saline (PBS, 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 dissolved in H_2O to a total volume of 1 l) and counted in a Coulter counter.

Detection of infectious retrovirus. Virus detection was attempted in 3 ways: from culture media in which the various cells were growing, from cell homogenates, and from intact epidermal cells by co-cultivation with target cells. Two target cell lines were used: normal mink lung (CCL64) and an S^+L^- derivative of 3T3 mouse cells (D56). The former has previously been used to isolate mouse xenotropic type C retroviruses (7), and the latter has been shown to be a suitable cell line for propagation of both N- and B-tropic ecotropic murine retroviruses (8). Replicate cultures were inoculated with sterile medium as virus-negative controls. Target cells were passaged at 7-day intervals for 6-8 passages. Virus replication was determined by assaying for the presence in the culture fluid of sedimentable viral DNA polymerase (reverse transcriptase) activity (9) immediately prior to cell passage.

Immunological assays. F_c-IgG receptors were determined by rosette assays (10) using sheep red blood cells generously provided by Mr. Paul Becker of the National Institute of Neurological and Communicative Disorders and Stroke and antiserum against sheep red blood cells (Cordis Laboratories, Miami, FL). Ia antigens were determined as previously described (10), with either polyspecific Ia antiserum (AT.H anti-A.TL) or with normal mouse serum as negative control, and the second antibody was fluorescein-isothiocyanate-conjugated rabbit anti-mouse IgG (Cappel Laboratories). The presence of keratin was similarly determined by indirect immunofluorescence microscopy using anti-keratin antisera kindly provided by Dr. Miriam Poirier, NCI. ATPase-positive cells were determined by the method of MacKenzie and Squier (11). Contact sensitivity to 2,4,6-trinitrochlorobenzene (Sigma Chemical Co., St. Louis, MO) was assessed as previously described (12). For the epidermal lymphocyte reaction, spleen cells from C57Bl/6 mice were used as responder T cells. Purified spleen T cells were exposed to 2000 rads of X-irradiation and incubated with primary epidermal cells to be tested. After 5 days, 10 μCi [^{3}H]thymidine (25 Ci/mmole, New England Nuclear, Boston, MA) were added to each well for the last 16 hr of culture.

RESULTS AND DISCUSSION

DNA repair and carcinogen binding.

One of the most striking genetically based skin cancer susceptibility conditions in humans is *Xeroderma pigmentosum*, in which there exist defects in ability to repair DNA damaged by UV irradiation and chemical carcinogens. Using the host cell reactivation assay previous used to characterize repair-defective Xeroderma cell lines, we examined the ability of primary epidermal cells of SENCAR, CD-1, Balb/c, and AKR mice to repair UV irradiated herpes simplex virus. These mouse strains form a spectrum of sensitivities to skin carcinogenesis from susceptible to resistant, respectively. Cells of all four strains were equally capable of reactivating irradiated virus (13) Furthermore, two non-tumorigenic Balb/c epidermal cell lines resistant to Ca^{2+}-induced terminal differentiation responded similarly. In a related study, we found similar levels of DNA polymerase β, the putative repair enzyme, in primary epidermal cells from Balb/c and SENCAR mice (Strickland, unpublished observations). Others have shown that binding of the activated carcinogen N-acetoxyacetylaminofluorene to SENCAR and Balb/c primary epidermal cells exposed *in vitro* was quantitatively and qualitatively similar (Dr. Miriam Poirier, personal communication). These results suggest that susceptibility of SENCAR epidermal cells to carcinogenesis is probably not accounted for on the basis of alterations in DNA repair or carcinogen binding.

Immunological factors

UV radiation-induced skin carcinogenesis in mice has been shown to involve activation of suppressor T cells that permit progressive growth of UV-induced tumors (14). In view of the results of carcinogenesis studies on grafted skin, we have examined two areas of immune responses which involve the epidermis.

In collaboration with Dr. Thomas Luger of the NCI, we measured epidermal thymocyte activating factor (ETAF) which is produced by keratinocytes and interacts with T-lymphocytes. Under a variety of conditions - with and without TPA, in medium with high and low Ca^{2+} levels - SENCAR primary epidermal cell cultures produced amounts of ETAF similar to those found in Balb/c cultures (Luger and Strickland, unpublished observations). Epidermal Langerhans cells (LC) are dendritic, suprabasal cells derived from bone marrow which play a major role in cell-mediated immune functions of the skin, including allergic contact sensitivity and antigen and alloantigen presentation. We detected similar numbers of LC in epidermis and fresh epidermal cell suspensions of both newborn and adult Balb/c and SENCAR mice by several independent analytical methods. However, in primary newborn epidermal cell cultures examined 24 hr after plating, there were more than 4X as many Fc-positive Balb/c cells as SENCAR. This difference probably results from SENCAR LC being less adherent to plastic than Balb/c LC, which suggests cell surface differences. Survival in culture of LC from the two strains was similar, with greatly enhanced survival in serum which had been heated at 56° for 30 min prior to addition to the growth medium. We have been unable to associate any functional significance with the presumed LC cell surface differences. Functional studies in adults, including contact sensitization and alloantigen presenting ability, showed no significant strain differences. Table 1 summarizes these immunological results. Topical treatment of skin in vivo or of cultured primary epidermal cells with compounds having tumor promoting activity such as TPA, teleocidin, and mezerein, lead to a marked increase in cells with Fc receptors (data not shown). Since these cells stain with antibody to Ia antigen but not with antikeratin antibody, we believe that tumor promoter treatment results in actual increased numbers of LC rather than induction of Fc receptors in other cell types. However, no significant strain differences have been found in the response of LC to tumor promoters. Therefore, we have been unable to implicate epidermal immune functions in the mechanism of SENCAR sensitivity to carcinogenesis.

In vitro growth properties.

We examined growth kinetics of epidermal cells from Balb/c and SENCAR under high and low Ca^{2+} conditions, in response to TPA, retinoic acid, epidermal growth factor (EGF), hydrocortisone, and fluocinolone acetonide by cell counts as well as by thymidine incorporation measurements on a daily basis for a time period after treatment. Basically, significant strain differences were not seen in effects of these agents on cell growth in culture (Fig. 1). Neither fluocinolone acetonide (Fig. 1B) nor hydrocortisone (not shown) at doses from 0.1 to 10 ug/ml affected proliferation of cells of either strain. Treatment with 100 ng/ml TPA led to an approximately 50% loss of attached cells in both strains, consistent with the induction of terminal differentiation in half the cells (15). Considerable emphasis was placed upon study of EGF interactions with cultured epidermal cells because of the possibility of a transforming-type growth factor produced by SENCAR cells and the modulating effects of TPA and retinoic acid [illegible] GF binding in other cell types. These studies are reported in ref. [illegible] The only significant strain difference found was in the rate of loss of EGF binding capacity after induction of terminal differentiation by raising Ca^{2+} levels in the medium. The decrease was consistently faster and greater in SENCAR cells compared to Balb/c. There is no obvious relationship between this property and SENCAR susceptibility, however. Otherwise, Balb/c and SENCAR primary epidermal basal cells responded similarly as follows: Over the range tested (10^4-10^5 cells/cm^2), EGF binding per cell increased 2-3 fold with increasing cell density. The two strains had similar numbers of receptors per cell. TPA treatment quickly (<30 min) and strongly (80-90% at 100 ng/ml) suppressed the ability of cells to bind EGF (Fig. 2), while treatment of epidermal basal cells with retinoic acid resulted in increased EGF binding (Fig. 2). Treatment with EGF at 1 and 10 ng/ml stimulated cell growth and resulted in higher saturation densities compared to controls without EGF. Cell growth was stimulated only in proliferating cultures. It was ineffective, for example, if cells were plated at very low density. Binding curves indicated that 36 h into the differentiation program induced by Ca^{2+}, cells bound lower levels and no longer metabolized EGF. Furthermore, differentiating cells were much less responsive to the modulation of EGF receptors by TPA and retinoic acid than were basal cells.

Virus and oncogene expression

We found spontaneous expression of endogenous xenotropic but not ecotropic type C RNA virus in epidermal cell lines from both SENCAR and Balb/c mice. This expression occurred at low levels, since infectious virus was demonstrable only by cocultivation of epidermal cells with appropriate target cells and not in homogenates or culture fluid from these same cells. Furthermore, xenotropic virus expression was not seen in SENCAR primary cells but only in a cell line resistant to Ca^{2+}-induced terminal differentiation, whereas virus was detected in primary Balb/c epidermal cells as well as lines with altered differentiation properties. Again, this virus was only detectible by co-cultivation for 4-6 weeks with target cells. Epidermal cells from Balb/c and SENCAR mice had similar levels of surface receptor for gp 70, the major murine leukemia virus (MuLV) envelope glycoprotein, and were good target cells for infection with Rauscher and AKR MuLV. These results are summarized in Table 2. Although expression of infectious endogenous retrovirus is highly repressed, we have, in collaboration with Dr. W. K. Yang of Oak Ridge National Laboratory, detected virus-related sequences in poly A RNA preparations from primary epidermal cells as well as lines derived from primaries by carcinogen treatment and selection for resistance to terminal differentiation (Yang and Strickland, unpublished observations). An RNA species with sequences homologous to the viral long terminal repeat (LTR), which contains elements that regulate gene expression, is expressed in several resistant cell lines but is absent from primary epidermal cells of both strains. The level of expression of a number of oncogenes did not differ significantly in SENCAR and Balb/c primary epidermal cells (Dr. Dennis Roop, NCI, personal communication). From the present data we cannot rule out expression of one or more of these oncogenes at normal levels but with an altered gene product in the cells tested.

Is SENCAR sensitivity related to initiation or to promotion

A major achievement in defining the mechanism of SENCAR sensitivity to skin carcinogenesis would be the determination of whether initiation, promotion, or both are involved. Promotion is clearly an important factor. The latent periods before the first papillomas and carcinomas appear in initiation-promotion studies is shorter, often by many weeks, in SENCAR than in resistant mouse strains. SENCAR skin is unusually sensitive to toxic effects of TPA. In the *in vivo* studies of Hennings *et al.* (2), SENCAR, but not Balb/c mice developed ulcerative skin lesions in response to TPA, necessitating reduction of treatments from twice to once per week. SENCAR epidermal cells have no more phorbol ester receptors than resistant strains (Dr. Peter Blumberg, NCI, personal communication), nor have we been able to identify any unusual biochemical response of cultured SENCAR cells to phorbol ester tumor promoters. The study of Strickland (3), reporting sensitivity of SENCAR mice to skin tumor development after a single dose of UV radiation, suggested UV might provide a chronic promotion stimulus because irradiated skin of SENCAR but not CD-1 controls "appeared raised and leathery for several months" after irradiation. _In vivo_ carcinogenesis studies from several laboratories have shown that mouse strains quite resistant to skin carcinogenesis by initiation-promotion may be very sensitive to tumor induction by multiple carcinogen treatment. This implies that resistance may be related to poor responsiveness to TPA, the commonly used promoter, and, conversely, sensitivity may be a consequence of high responsiveness to TPA. A recent report (17) suggests that resistance or sensitivity to initiation-promotion may vary depending upon the promoting agent used. Recent studies in our laboratory have provided strong support for a relationship between initiation and resistance to Ca^{2+}-induced terminal differentiation (18). In direct comparisons of the yield of Ca^{2+}-resistant foci from Balb/c and SENCAR epidermal cells initiated either _in vivo_ or in culture by DMBA or MNNG, little, if any, difference between strains in number of foci obtained for a given treatment has been found. Notable was the frequent presence of foci in untreated dishes of SENCAR but rarely Balb/c cells, consistent with the formation of tumors in SENCAR mice treated with TPA alone in the absence of exogenous initiation. In spite of the absence of quantitative strain differences in carcinogen-induced, Ca^{2+}-resistant foci, there are qualitative differences which may provide useful clues but have been difficult to quantify. Although some SENCAR foci resemble those seen in Balb/c cultures, others have a different appearance and behavior. In general, it has been much easier to obtain cell lines from SENCAR than from Balb/c foci due to differences in survival after subculture. The inducibility of ornithine decarboxylase by TPA and transglutaminase by retinoic acid was generally greater in cell lines derived from SENCAR than from Balb/c foci. A detailed comparison of properties of these cell lines is in progress (Kawamura *et al.*, in preparation). The resistant cell lines obtained from SENCAR foci differ in some responses to TPA in culture from cell lines derived from papillomas on SENCAR mice. TPA treatment of papilloma cell lines but not all Ca^{2+}-resistant lines leads to stimulation of DNA synthesis and enhanced cloning efficiency (Yuspa *et al.*, in preparation).

In conclusion, the biological and biochemical responses of SENCAR and Balb/c primary epidermal cells in culture in a variety of areas, including DNA repair, immune functions, growth in the presence of modulators of carcinogenesis, and binding and metabolism of EGF, have been remarkably similar. We have yet to determine whether these similarities will persist if cultured cells are subjected to repeated challenge with TPA in a manner similar to carcinogenesis protocols *in vivo*. The low levels of spontaneous expression of infectious xenotropic virus by Balb/c but not SENCAR primary cells is probably of no consequence because of the long periods (4-6 weeks) of co-cultivation necessary to detect the difference. Oncogene expression was not significantly different in Balb/c and SENCAR cells. Although evidence from both *in vivo* and *in vitro* studies points to the existence of a population of constitutively initiated epidermal cells in SENCAR which is absent or greatly reduced in Balb/c skin, the role of such cells in SENCAR sensitivity has not been well defined. However, we have provided evidence that in response to the exogenous chemical initiators MNNG and DMBA Balb/c cells are quantitatively equally as well initiated as SENCAR. We are presently characterizing what we believe are initiated cells, especially with reference to their response to promoters and their potential for conversion to malignancy. These cells and their response to promoters may provide the key to SENCAR sensitivity.

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TABLE 1

Summary of immunological properties of epidermal cells from SENCAR and Balb/c mice

	Balb/c	SENCAR
Langerhans cell density in epidermis (cell/mm^2)		
ATPase staining	714 ± 153	696 ± 142
Ia antigen staining	383 ± 40	375 ± 88
% Fc-positive cells in fresh epidermal cell suspensions	2.9	2.7
Alloantigen presenting ability[a] (cpm)	3,321 ± 384	3,158 ± 265
Allergic contact sensitization[b] (10^{-2} x mm)	6.0 ± 1.2	4.9 ± 2.0

All values are means ± standard errors of the mean.

[a] [^{3}H]thymidine incorporation by 2 x 10^5 T-cells from C57Bl/6 mice co-cultured 5 d with 5 x 10^4 irradiated Balb/c or SENCAR epidermal cells. Background was 689 ± 81 cpm.

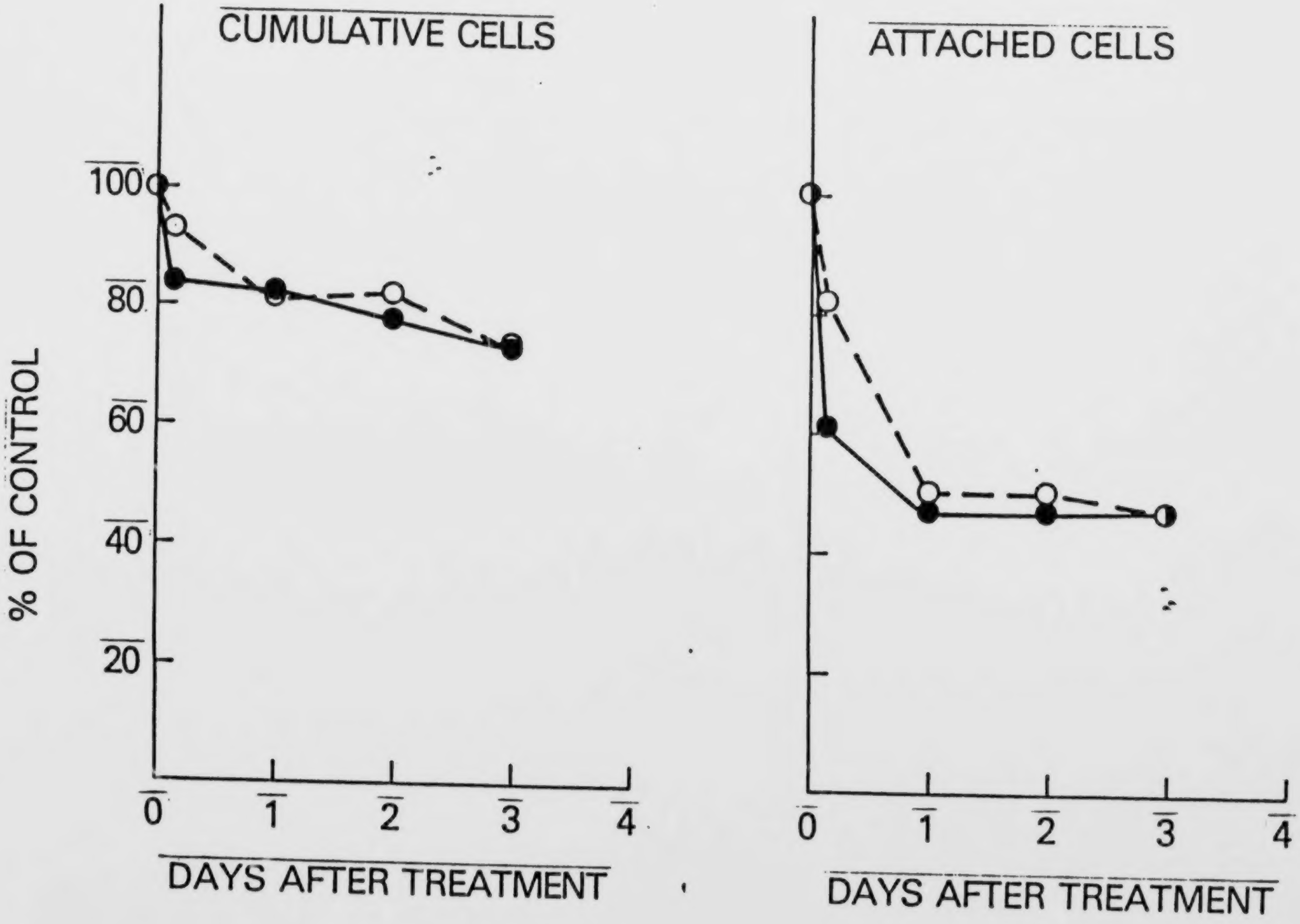
[b] Increment in ear swelling upon application of 1% 2,4,6-trinitrochlorobenzene after sensitization 6 d previously (corrected for negative control).

TABLE 2

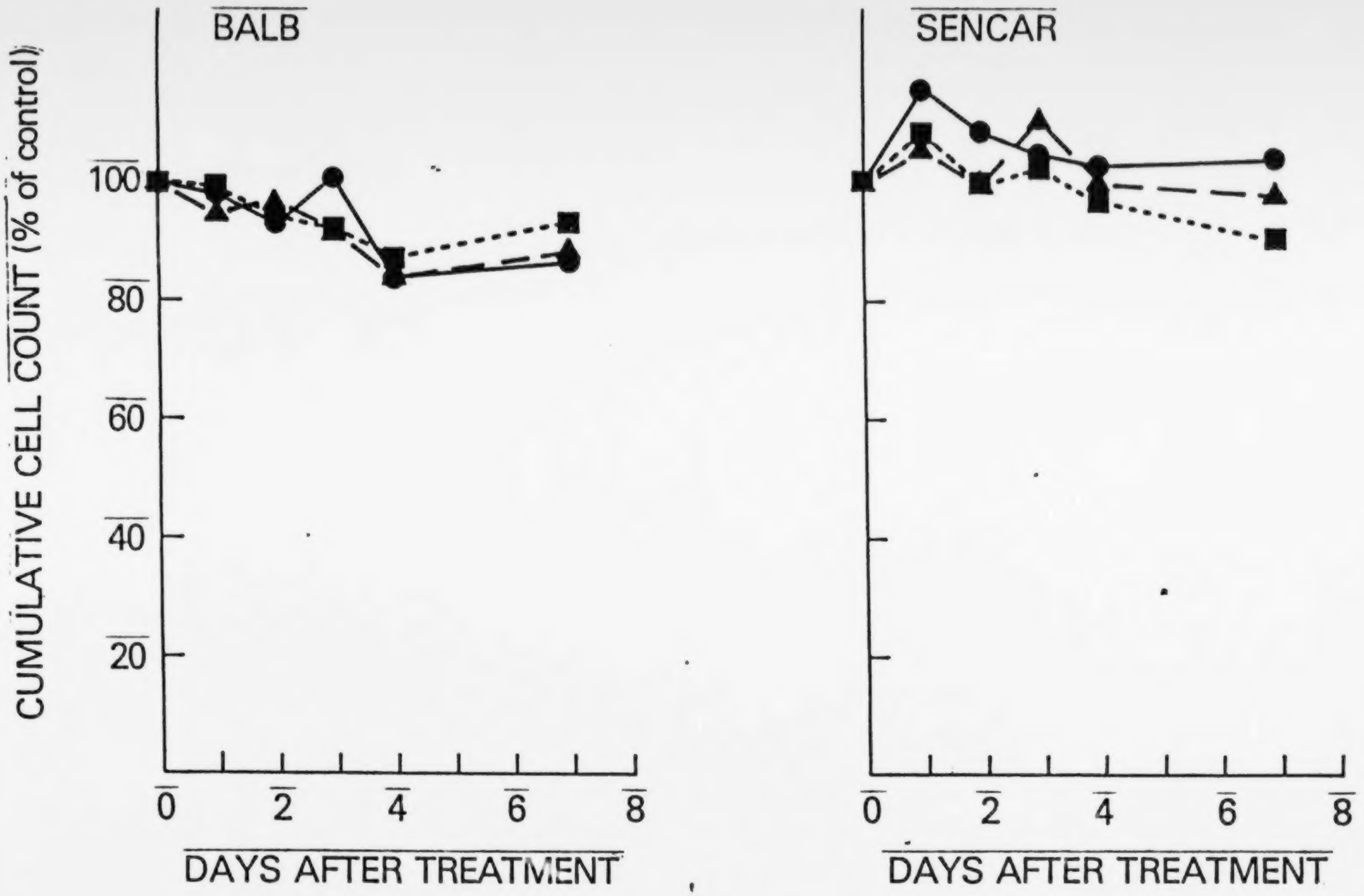
Retroviruses and mouse epidermal cells: expression and infection

	Balb/c	SENCAR
Expression of endogenous infectious retrovirus		
in primary cells	+	-
in cell lines resistant to Ca^{2+}-induced terminal differentiation	+	+
Presence of receptors for virus gp 70	+	+
Infectibility by R-MuLV and AKR MuLV	+	+

Figure 1. Growth of cultured primary newborn epidermal cells in the presence of TPA (A) or fluocinolone acetonide (B). Two X 10^6 cells were plated in 60-mm dishes. Medium with 0.05 mM Ca^{2+} was changed daily, and treatment was begun 6 days after plating with TPA or fluocinolone acetonide subsequently present throughout. Attached and unattached cells were counted at the times indicated. Cumulative cells were determined by summing the number of new cells each day with the number of attached cells at the beginning of treatment. (A) Cumulative and attached Balb/c (—●—) and SENCAR (--o--) cells treated with 100 ng/ml TPA, expressed as % of solvent (0.1% DMSO)-treated controls. (B) Cumulative cell counts of Balb/c and SENCAR cells exposed to 0.1 (●), 1.0 (▲), or 10 (■) µg/ml fluocinolone acetonide, expressed as % of solvent (0.1% DMSO) control.

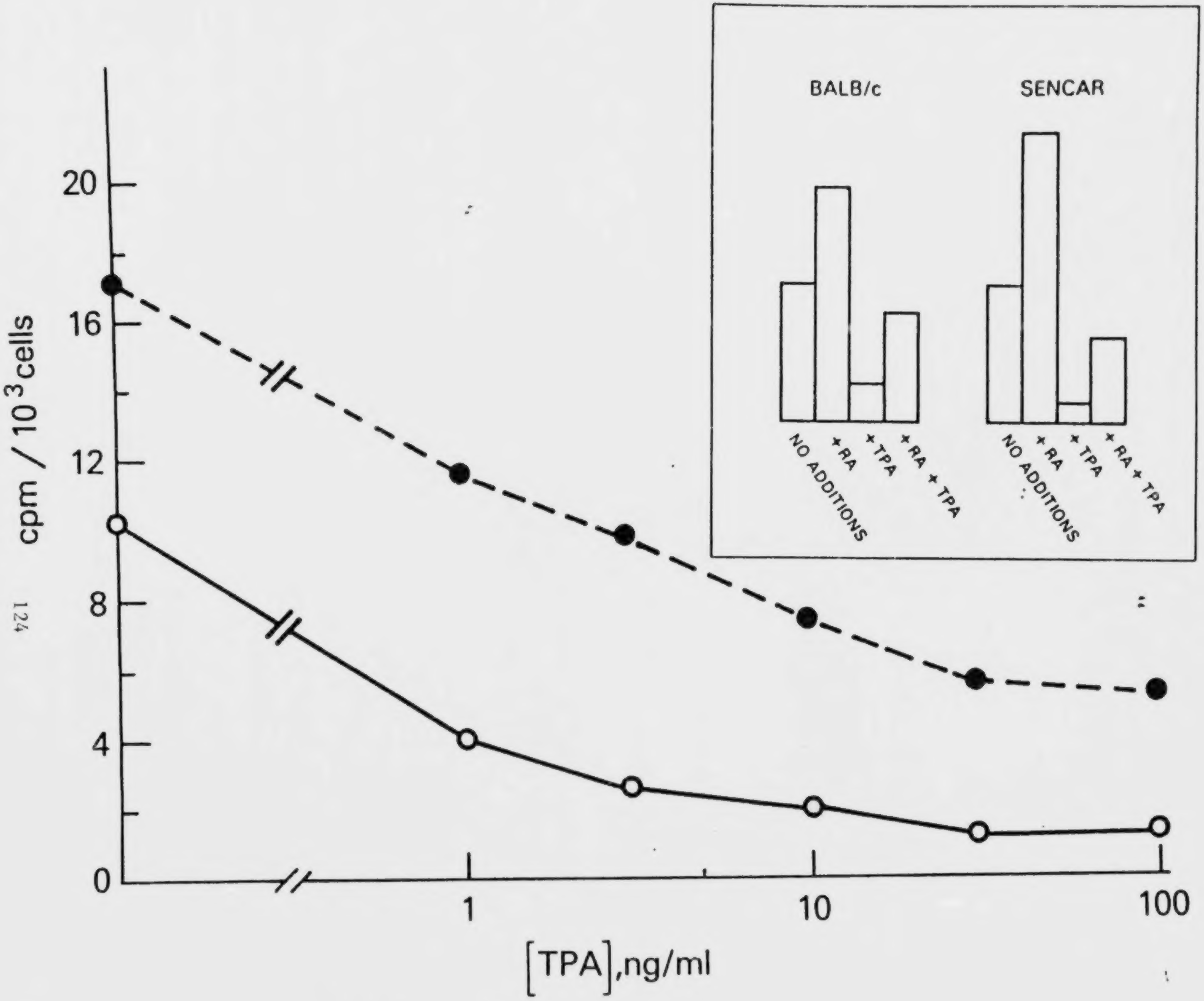

CUMULATIVE CELLS
ATTACHED CELLS
% OF CONTROL
100
80
60
40
20
0
1
2
3
4
DAYS AFTER TREATMENT
DAYS AFTER TREATMENT

A


BALB
SENCAR
CUMULATIVE CELL COUNT (% of control)
100
80
60
40
20
0
2
4
6
8
DAYS AFTER TREATMENT
0
2
4
6
8
DAYS AFTER TREATMENT

B

Figure 2. Dose response curve for the reduction in EGF binding in Balb/c primary epidermal basal cells by treatment (2.5 h at 37°) with TPA in the presence (--●--) or absence (—o—) of pretreatment with retinoic acid (10^{-5} M, 72 h at 37°). The inset shows comparative EGF binding in Balb/c and SENCAR primary epidermal basal cells. TPA treatment was 10 ng/ml, 2.5 h, and retinoic acid treatment was as above. The binding assay was incubated for 6 h at 0°. Total cell-associated radioactivity values were normalized to controls.


BALB/c
SENCAR
NO ADDITIONS
+ RA
+ TPA
+ RA + TPA
NO ADDITIONS
+ RA
+ TPA
+ RA + TPA
cpm / 10³ cells
20
16
12
8
4
0
1
10
100
[TPA], ng/ml

METABOLIC ACTIVATION OF BENZO(A)PYRENE IN SENCAR AND BALB/c MOUSE EMBRYO CELL CULTURES

by

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Key Words

benzo(a)pyrene
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benzo(a)pyrene diol-epoxide
DNA adducts
mouse embryo cell cultures
metabolism
glucuronide conjugates
immobilized boronate chromatography
high-performance liquid chromatography

ABSTRACT:

The metabolism and DNA-binding of benzo(a)pyrene (B(a)P) were compared in early passage mouse embryo cell cultures prepared from SENCAR mice, a strain especially susceptible to two-stage tumorigenesis, and BALB/c mice, a strain relatively resistant to two-stage tumorigenesis. Cultures from both strains metabolized similar amounts of B(a)P, however the proportion of water-soluble metabolites formed was higher in the BALB/c cultures than in the SENCAR cultures. The major metabolites formed in cultures from both strains were B(a)P-9,10-diol, B(a)P-7,8-diol and the glucuronic acid conjugate of 3-hydroxy-B(a)P. The level of binding of B(a)P to DNA was greater in the SENCAR mouse embryo cell cultures than in the BALB/c cultures after 5, 24 and 48 hours of exposure. The major B(a)P-DNA adduct formed in B(a)P-treated cultures from both strains was the adduct formed by reaction of (+)anti-B(a)P-7,8-diol-9,10-epoxide (anti-B(a)PDE; the isomer with the epoxide and the benzylic hydroxyl on opposite faces of the molecule) with the exocyclic amino group of deoxyguanosine. Immobilized boronate chromatography followed by high-performance liquid chromatography demonstrated the presence of small amounts of syn-B(a)PDE (the isomer with the epoxide and benzylic hydroxyl on the same face of the molecule)-DNA adducts: the proportions of these were similar in cultures from both strains. The results suggest that SENCAR mouse embryo cell cultures may convert less B(a)P to water-soluble metabolites and more to DNA-binding metabolites than BALB/c mouse embryo cells. If similar differences exist in B(a)P metabolism in the skin of these strains of mice, it could help to explain the relatively high sensitivity of the SENCAR mice to tumor induction by the two-stage protocol.

INTRODUCTION:

The susceptibility of rodents to tumor induction by carcinogenic chemicals is dependent upon both the species and strain of rodent as well as the particular type of carcinogen and the route of administration. The two-stage initiation-promotion protocol in mouse skin has been used for over 40 years as an assay for detecting tumor initiators and promoters (1). The speed and sensitivity of this assay were greatly increased by Boutwell and coworkers through breeding of strains selected for susceptibility to initiation by 7,12-dimethylbenz(a)anthracene and promotion by croton oil or its most active component, 12-O-tetradecanoylphorbol-13-acetate (1,2). The SENCAR mice developed by this procedure are much more susceptible to tumor induction by this protocol than other mice such as BALB/c (3). Slaga et al. (4) have shown that a number of strains differ in their response to this treatment protocol and that SENCAR mice are much more susceptible than BALB/c mice to both complete and two-stage epidermal carcinogenesis. One component of this difference may be susceptibility to promotion (5), but there is also evidence for strain-dependent differences in the activation of the initiator. Nebert et al. (6) found that the induction of hydrocarbon metabolizing enzymes as well as tumor susceptibility varied in DBA/2 and C57Bl mice. The strain-dependent differences in these enzymes were recently reviewed by Pelkonen and Nebert (7).

Polycyclic aromatic hydrocarbons such as the environmental pollutant benzo(a)pyrene [B(a)P] require metabolic activation to induce biological effects (reviewed in 8,9). In order to trap the metabolites formed from B(a)P, most studies were carried out either with microsomal fractions from various tissues or in cells in culture (8). Early passage rodent embryo cell cultures were found to have high B(a)P metabolizing activity (10) and mouse embryo cell cultures were used to study the metabolism and DNA binding of B(a)P and other hydrocarbons (11,12). The first successful method for chromatographic isolation of B(*a*)P-DNA adducts was developed with DNA from B(*a*)P-treated mouse embryo cell cultures (13). Recently, techniques have been developed to culture mouse epidermal keratinocytes and to investigate the metabolism and DNA-binding of B(*a*)P in those cells (14,15,16,17). Although studies have been carried out in cells from a number of strains of mice, B(*a*)P metabolism and B(*a*)P-DNA adduct formation have not been compared directly in cells from various strains under identical conditions in order to assess the role of these factors in the susceptibility of various strains to tumor induction. The present study compared the metabolism of B(*a*)P in early passage embryo cell cultures prepared from SENCAR, a sensitive strain of mice in the two-stage carcinogenesis assay, and BALB/c, a relatively resistant strain of mice in this assay (3). The activation of B(*a*)P to DNA-binding metabolites was also investigated in cells from these strains by the use of immobilized-boronate chromatography followed by high-performance liquid chromatography (HPLC) (18), a procedure which allows increased resolution of the B(*a*)P-DNA adducts present.

MATERIALS AND METHODS:

Treatment of Cells. Primary embryo cell cultures were prepared from 15th-day SENCAR or BALB/c mouse embryos (Harlan Sprague-Dawley, Inc., Indianapolis, IN) as described previously (18). Tertiary monolayer cultures were prepared in 175 sq cm culture flasks with 50 ml minimum Eagle's medium (Grand Island Biological Co., Grand Island, NY) containing 10% fetal bovine serum (Reheis Chemical Co., Phoenix, AZ). $[G-^3H]$B(a)P (Amersham, Arlington Heights, IL) was diluted with B(a)P (Gold Label; Aldrich Chemical Co., Milwaukee, WI) to a specific activity of 5 Ci/mmole and added to the cultures at a final concentration of 0.5 μg/ml medium.

Benzo(a)pyrene Metabolism. After exposure of cells for 5, 24 and 48 hours, medium samples were removed, extracted by a 2-step chloroform:methanol:water procedure (19), and the amount of radioactivity in aliquots of each phase was determined by liquid scintillation counting. To measure the formation of hydrocarbon-glucuronides, medium samples were incubated with 2000 Fishman units of bovine liver β-glucuronidase prior to extraction (19,20). The chloroform phases were analyzed by HPLC on a Beckman Model 312 chromatograph equipped with a 4.6 mm x 25 cm Ultrasphere octadecyl silane (5 μm) reverse-phase column (Beckman Instruments, Inc., St. Louis, MO). Samples were eluted at 1 ml/min for 40 min with a linear methanol:water gradient from 11:9 to 17:3 followed by 10 min at 17:3 (20). One hundred ten fractions (0.5 ml) were collected, and radioactivity was determined by liquid scintillation counting. Reference standards of B(a)P metabolites were purchased from the Chemical Repository, Carcinogenesis Research Program, National Cancer Institute.

Analysis of B(a)P-DNA Adducts. After 5, 24 and 48 hours of exposure to B(a)P, the cells were harvested with trypsin:versene, and the nuclei were isolated by homogenization of the pellet in a hypotonic buffer containing Triton-X and stored at -80° (18). The DNA was isolated by treatment with RNase A (Sigma Chemical Co., St. Louis, MO) and proteinase K (Sigma) followed by $CHCl_3$:isoamyl alcohol (24:1, v/v) extraction and ethanol precipitation. After 3 repetitions, the DNA was precipitated first with 2-ethoxyethanol and then with ethanol (18). The DNA was enzymatically degraded to deoxyribonucleosides (13). B(*a*)P-deoxyribonucleoside adducts were isolated by chromatography on a Quick-Sep Sephadex LH-20 column (Isolab Inc., Akron, OH) (21). One portion was analyzed by HPLC and the remainder was analyzed by boronate chromatography prior to HPLC.

HPLC analyses were carried out on a 25 cm x 4.6 mm Ultrasphere octyl reverse-phase column (Beckman Instruments, Inc., St. Louis, MO). The column was eluted with methanol:water (46:54) for 34 min at a flow rate of 1.0 ml/min, then for 10 min with a linear gradient of methanol:water (46:54 to 55:45), followed by 24 min with methanol:water (55:54) (18). Fifteen 1.0 ml fractions followed by 145 fractions of 0.3 ml each were collected in scintillation vials. Radioactivity was determined by liquid scintillation counting using ACS (Amersham, Arlington Heights, IL). An aliquot of [^{14}C](+)*anti*-B(*a*)PDE (the isomer of B(*a*)P-7,8-diol-9,10-epoxide with the epoxide and benzylic hydroxyl on opposite faces of the molecule)-deoxyguanosine (dG) adduct, prepared by reaction of [^{14}C]*anti*-B(*a*)PDE (National Cancer Institute, Radiochemical Repository) with calf thymus DNA as described previously (21), was added to each sample as a marker.

B(*a*)P-deoxyribonucleoside adduct samples for boronate chromatography were chromatographed on a 1.0 g column of ((N-(N'-[m-(dihydroxyboryl)phenyl]succinamyl)amino))ethyl cellulose as described previously (18). The column was eluted with 40 ml of 1 M morpholine (pH 9.0), followed by 40 ml of 1 M morpholine:10% sorbitol, pH 9.0. Eighty 1.0 ml fractions were collected, and 0.1 ml aliquots of all fractions were removed and placed in scintillation vials. Radioactivity was determined by liquid scintillation counting in Ready-Solv HP (Beckman Instruments, Inc. Fullerton, CA). Buffer fractions containing radioactivity were pooled, applied to a Quik-Sep Sephadex LH-20 column, and the B(a)P-modified deoxyribonucleosides were eluted with methanol (18).

RESULTS:

The metabolism of benzo(a)pyrene was compared in confluent third passage cultures of SENCAR and BALB/c mouse embryo cells. The cultures were exposed to [^{3}H]B(a)P and aliquots of media were removed at 5, 24 and 48 hours for measurement of metabolism. The samples were extracted with chloroform:methanol and the chloroform phase analyzed by HPLC to determine the amount of B(a)P metabolized. The percentages of the B(a)P metabolized are shown in Fig. 1. The BALB/c cells appeared to metabolize slightly more B(a)P at all three time points, but none of the differences were statistically significant. Most of the B(a)P was metabolized by cultures from both strains of mice by 24 hours.

The nature of the metabolites formed was investigated by determining the proportion of the metabolites that remained in the aqueous-methanol phase after chloroform extraction. The percentage of water-soluble metabolites at each time point is shown in Fig. 2. At all times, the proportion of water-soluble metabolites was higher in the BALB/c than in the SENCAR cells; the increased level in the BALB/c samples was statistically significant in both the 5 and 48 hour samples. The chloroform-extractable metabolites were analyzed by HPLC and the metabolite profiles are shown in Fig. 3. The major metabolites present in cultures from both strains were the 9,10-diol and the 7,8-diol with only traces of phenolic metabolites present. The percentages of the total metabolites that these two diols and two phenols represent are shown in Fig. 4. At the early times, 5 and 24 hours, the SENCAR cell medium contained a higher proportion of 9,10-diol than the BALB/c medium. In both cell types the highest proportion of these primary metabolites was observed at 5 hours, then this percentage dropped to a very low level by 24 and 48 hours, presumably due to further metabolism of the primary metabolites.

To investigate the nature of the water-soluble metabolites formed in the cultures from these two strains of mice, medium samples were treated with β-glucuronidase to cleave glucuronide conjugates prior to chloroform extraction. The HPLC profiles of these extracts (Fig. 5) show the presence of three additional peaks, a broad peak in the quinone region (Fractions 50-65) and peaks that chromatographed with markers of 9-hydroxy-B(*a*)P and 3-hydroxy-B(*a*)P. The 3-hydroxy-B(*a*)P peak was much larger than the 9-hydroxy-B(*a*)P peak in cells from both strains and the ratio of 9- to 3-hydroxy B(*a*)P was similar in both.

The binding of B(*a*)P to DNA was investigated at 5, 24 and 48 hours. At all times the SENCAR cells contained a larger amount of B(*a*)P bound to DNA than the BALB/c cells (Fig. 6). Binding increased from 5 to 24 hours and continued to increase from 24 to 48 hours in spite of the fact that almost 80% of the B(*a*)P had been metabolized by 24 hours.

The B(*a*)P-DNA adducts present were then analyzed by HPLC (Fig. 7). Cells from both strains contained one large adduct peak that coeluted with the adduct formed by reaction of [^{14}C]*anti*-B(*a*)PDE with the exocyclic amino group of deoxyguanosine (*anti*-B(a)PDE-dG) (reviewed in 9). In addition, several smaller adduct peaks were present, but some were only partially resolved from the *anti*-B(*a*)PDE-dG peak. To allow the resolution of these minor adduct peaks, the B(*a*)P-DNA adducts were separated into those that contained *cis* vicinal hydroxyl groups (such a those formed from *anti*-B(a)PDE) and those that did not contain *cis* vicinal hydroxyl groups (such as those formed by *syn*-B(a)PDE, the isomer with the epoxide and benzylic hydroxyl on the same face of the molecule) by immobilized boronate chromatography. The column profiles are shown in Fig. 8. Although some material eluted in the morpholine buffer fractions (1-20), the major portion of the adducts eluted after the addition of sorbitol to the buffer (arrow). Thus, the majority of the DNA adducts in both SENCAR and BALB/c mouse embryo cells resulted from binding of the *anti*-isomer of B(*a*)PDE to DNA. The ratio of sorbitol buffer-eluted material to morpholine buffer-eluted material (*anti*- to *syn*-B(*a*)PDE-DNA adducts) is shown in Fig. 9. The ratio increased from less than 2:1 to more than 5:1 in both cell types between 5 and 48 hours, but there was no significant difference in the ratio present in BALB/c cultures from that present in SENCAR cell cultures at any time.

The individual B(*a*)P-DNA adducts present in the morpholine and sorbitol buffer fractions were analyzed by HPLC on a reverse-phase C_8 column. The profiles for the BALB/c culture DNA samples are shown in Fig. 10. The sorbitol buffer fractions contained one major peak that coeluted with the $[^{14}C]$(+)*anti*-B(*a*)PDE-dG marker and only very small amounts of three other adducts. The one in fraction 140 elutes in the same position as an *anti*-B(*a*)PDE-deoxyadenosine marker; the others are unidentified. Although the morpholine buffer fractions contained a much smaller proportion of the total material, this material did contain three distinct peaks of adducts. The first (fractions 92-98), which was only partially resolved from the *anti*-B(*a*)PDE-dG peak by HPLC alone (Fig. 7), eluted in the same position as *syn*-B(*a*)PDE-dG and *syn*-B(*a*)PDE-deoxycytidine markers. The largest peak (fraction 116-121) eluted in the same position as a marker of *syn*-B(*a*)PDE-dG and the small third peak eluted in the same relative position as *syn*-B(*a*)PDE-deoxyadenosine. The HPLC profiles from immobilized boronate column fractions of the B(*a*)P-DNA adducts from the SENCAR mouse embryo cell cultures (Fig. 11) contained the same adduct peaks in almost identical proportions. Previous studies have demonstrated that the relative proportions of the three peaks within the morpholine buffer fraction do not change with time. The major time-dependent change in the adducts is the increase in the proportion of the (+)*anti*-B(*a*)PDE-dG adduct which is responsible for the time-dependent increase in the ratio of *anti*- to *syn*-B(*a*)PDE-DNA adducts (Fig. 9).

DISCUSSION:

To determine whether strain-specific differences in the metabolic activation of B(a)P could help to explain the difference in tumor susceptibility of BALB/c and SENCAR mice, B(a)P metabolism and DNA binding were investigated in embryo cell cultures prepared from SENCAR and BALB/c mice. The metabolism studies demonstrated that both the SENCAR and BALB/c mouse embryo cell cultures metabolized similar amounts of B(a)P at all three times studied. The only difference in B(a)P metabolism between the cells from the two strains was the higher proportion of water-soluble metabolites in the BALB/c cultures. This suggests that the BALB/c cells may metabolize the primary diols and phenols more rapidly and reduce the proportion activated to DNA-binding derivatives. The organic solvent extractable B(a)P metabolites in both cells were similar to those found in SENCAR mouse epidermal cell cultures by DiGiovanni *et al*. (15). The relative ratio of 9,10-diol to 7,8-diol was greater in the embryo cell cultures than in the SENCAR epidermal cell cultures (15), but similar to that observed in two epidermal cell lines (15). β-glucuronidase treatment released mainly 3-hydroxy-B(a)P and a small amount of 9-hydroxy-B(a)P in both SENCAR and BALB/c mouse embryo cell cultures, a result identical to that observed in SENCAR epidermal cell cultures (15). Thus, B(a)P metabolism in the mouse embryo cell cultures was similar to that reported for epidermal cell cultures. The pattern of B(a)P metabolism in mouse embryo cell cultures differed from that observed in Syrian hamster and Wistar rat embryo cell cultures, for cultures from both of those species formed a higher proportion of 9-hydroxy-B(a)P glucuronide conjugates (22).

The binding of hydrocarbons to DNA in mouse skin and in mouse embryo cell cultures has been shown to correlate with the carcinogenic potential of the hydrocarbon (11,23). In studies of B(a)P-DNA binding in various strains of mice however, Phillips *et al.* (24) found no significant differences in the binding of B(*a*)P to DNA in strains of varying sensitivity to hydrocarbon carcinogenesis. The level of binding of B(*a*)P to DNA was greater at all times in the SENCAR mouse embryo cells than in the BALB/c mouse embryo cells, a result that correlates with relative susceptibility of these two strains to tumor induction. The actual B(*a*)P-DNA adducts formed in both are similar, the (+)*anti*-B(*a*)PDE-deoxyguanosine adduct was the major adduct at all times. There were also small amounts of two *syn*-B(*a*)PDE-deoxyribonucleoside adducts and *syn*- and *anti*-B(*a*)PDE-deoxyadenosine adducts, but the relative proportions of these were almost identical in cells from both strains. In both the SENCAR and the BALB/c cell cultures there was a time-dependent increase in the proportion of the *anti*-B(*a*)PDE-DNA adducts, a change that resulted from the increase in the (+)*anti*-B(*a*)PDE-dG adduct. The profile of adducts formed in the SENCAR mouse embryo cells was very similar to that reported by Ashurst *et al.* (25) for SENCAR mouse epidermis *in vivo*. The high proportion of the (+)*anti*-B(*a*)PDE-dG adduct was also consistent with the antibody binding studies of Nakayame *et al.* (16) in BALB/c epidermis *in vivo*. Thus the binding of B(a)P to DNA in mouse embryo cell cultures appears to be similar to B(*a*)P-DNA binding in mouse skin.

The B(*a*)P-DNA adduct profiles in the mouse embryo cell cultures differed dramatically from those formed in Syrian hamster and Wistar and Fisher rat embryo cell cultures (26). Both the rat and hamster cells contained a much more complex profile of adducts and exhibited greater time-dependent alterations in the adducts present. The amount of (+)*anti*-BaPDE-dG adduct present in the mouse embryo cell cultures at 24 hours was much greater than that found in either the rat or the hamster embryo cell cultures (26). The (+)enantiomer of *anti*-B(*a*)PDE is the most potent skin tumor initiator of the B(*a*)PDE enantiomers (27). The results suggest that both SENCAR and BALB/c mouse embryo cell cultures form similar proportions of the major-DNA adduct of

(+)anti-B(a)PDE, but the higher level of binding of B(a)P to DNA in the SENCAR mouse embryo cells may result from formation of higher amounts of this ultimate carcinogen in SENCAR cells than in BALB/c cells. If this also occurs in epidermis, it could help to explain the increased susceptibility of SENCAR mice to two-stage carcinogenesis.

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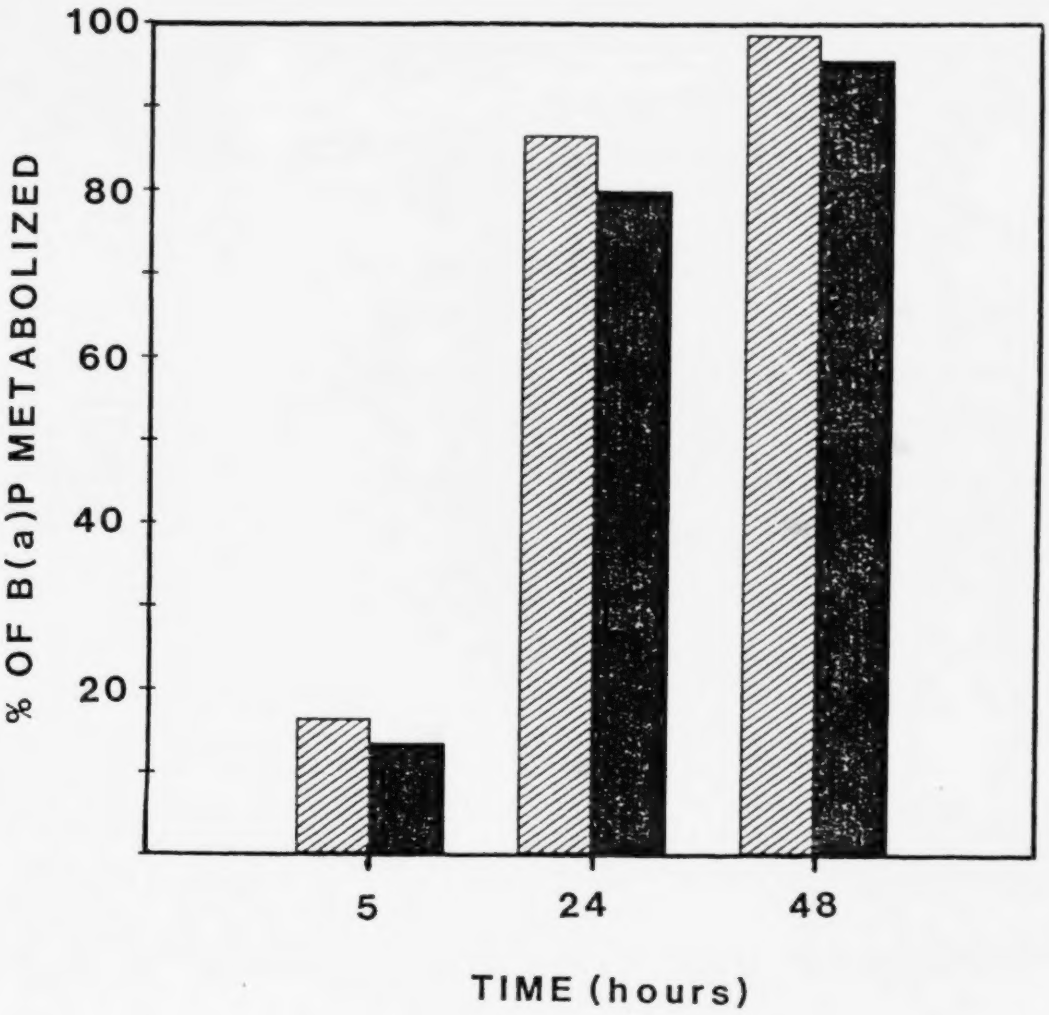

Figure 1. Percentage of B(*a*)P metabolized by BALB/c and SENCAR mouse embryo cell cultures. Cultures were exposed to 0.5 μg [^{3}H]B(*a*)P for the times stated, medium samples were removed, extracted with chloroform:methanol and the percentage of B(*a*)P remaining determined by HPLC. ▨ BALB/c cell cultures. ■ SENCAR cell cultures.

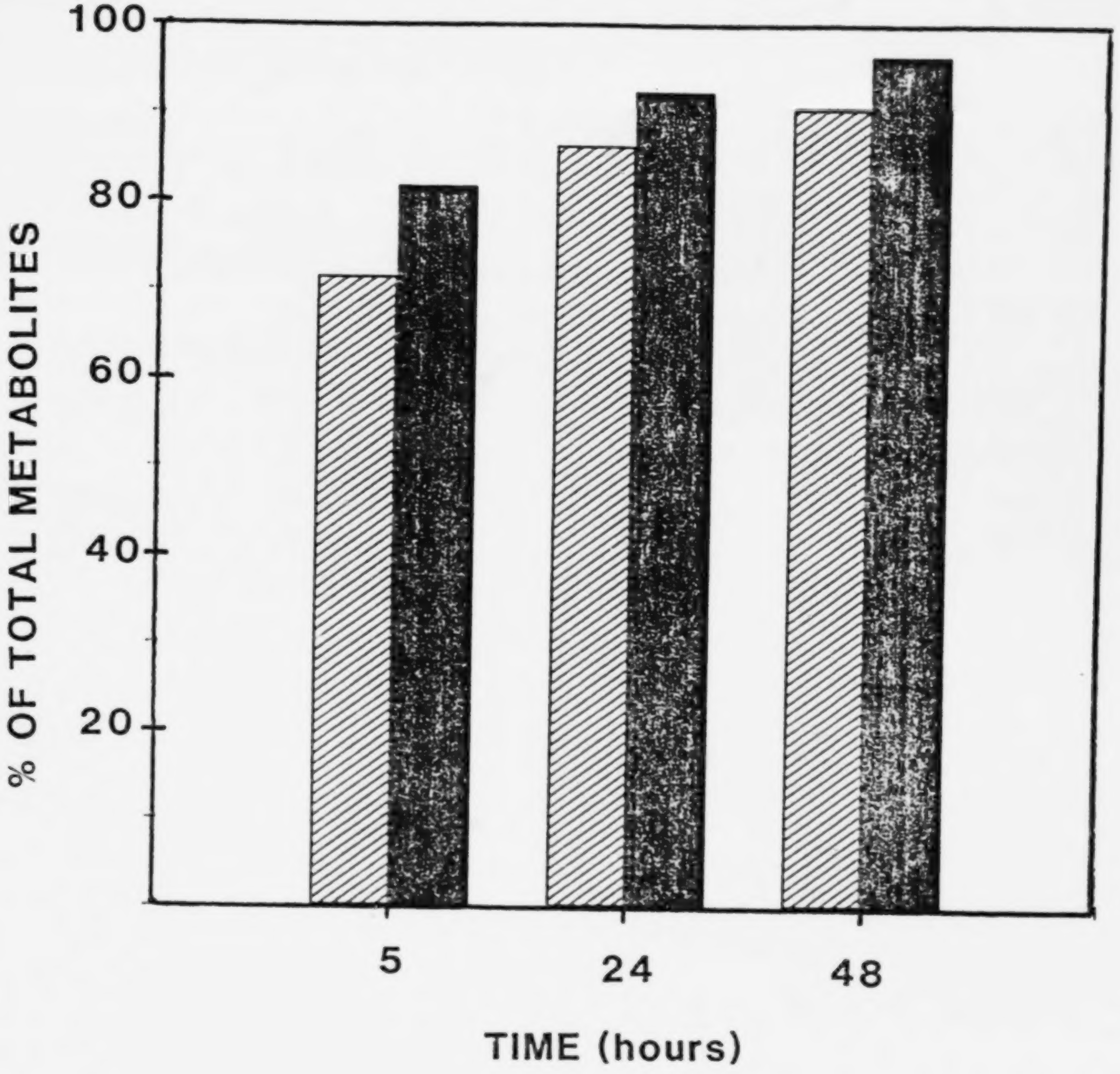

Figure 2. Percentage of water-soluble B(a)P metabolites formed in BALB/c and SENCAR mouse embryo cell cultures. Cultures were treated with $[^3H]$B(a)P and analyzed as described in the legend of Fig. 1. The percentage of the total B(a)P metabolites recovered in the aqueous phase was calculated. ▨ SENCAR cell cultures. ■ BALB/c cell cultures.

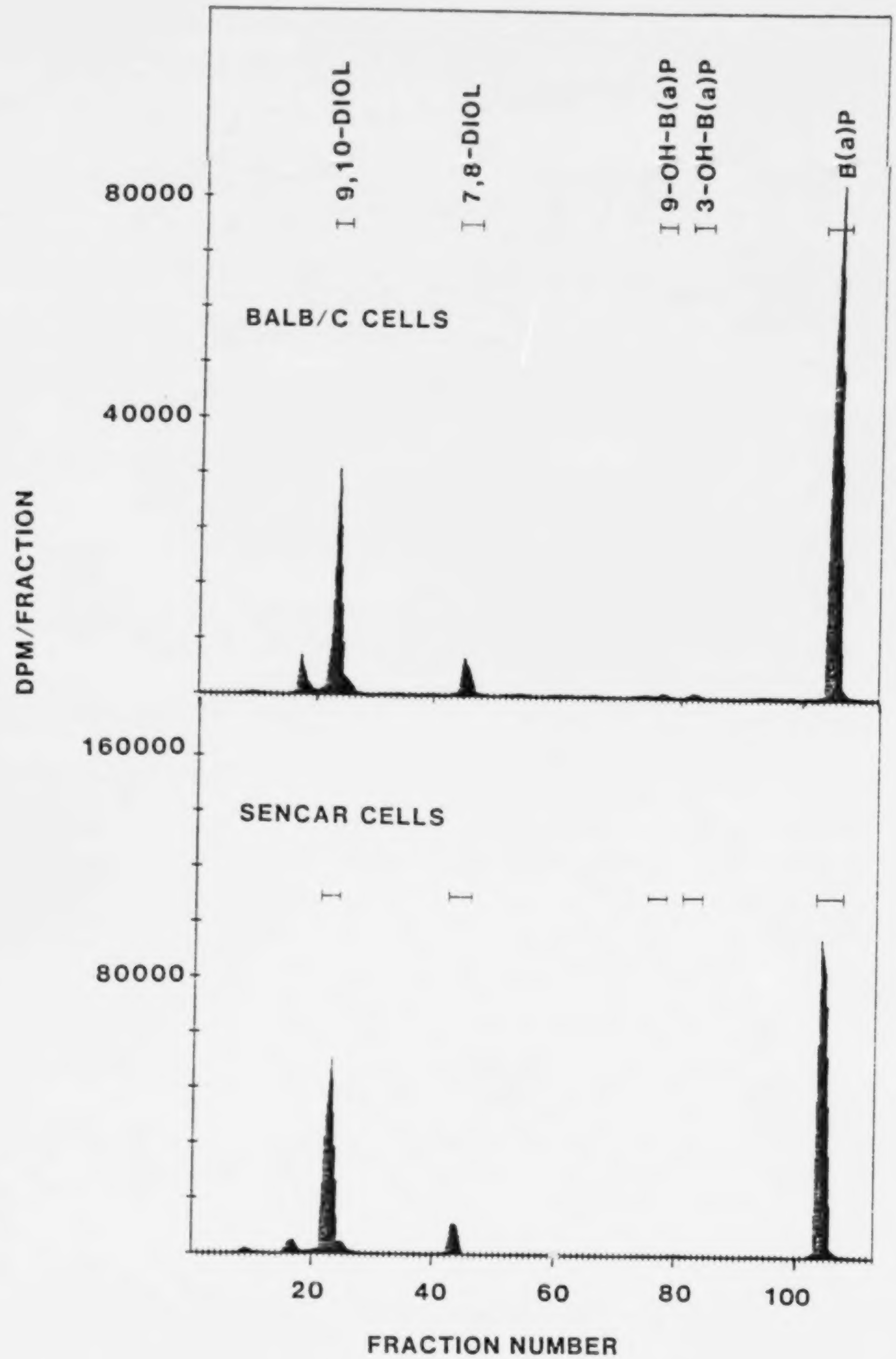

Figure 3. HPLC elution profile of the chloroform-extractable B(a)P metabolites formed in BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. Cultures were exposed to 0.5 μg [^{3}H]B(a)P/ml medium for 24 hours and extracted and chromatographed as described in Materials and Methods. The elution positions of B(a)P metabolite markers are shown by the ⊢⊣ .

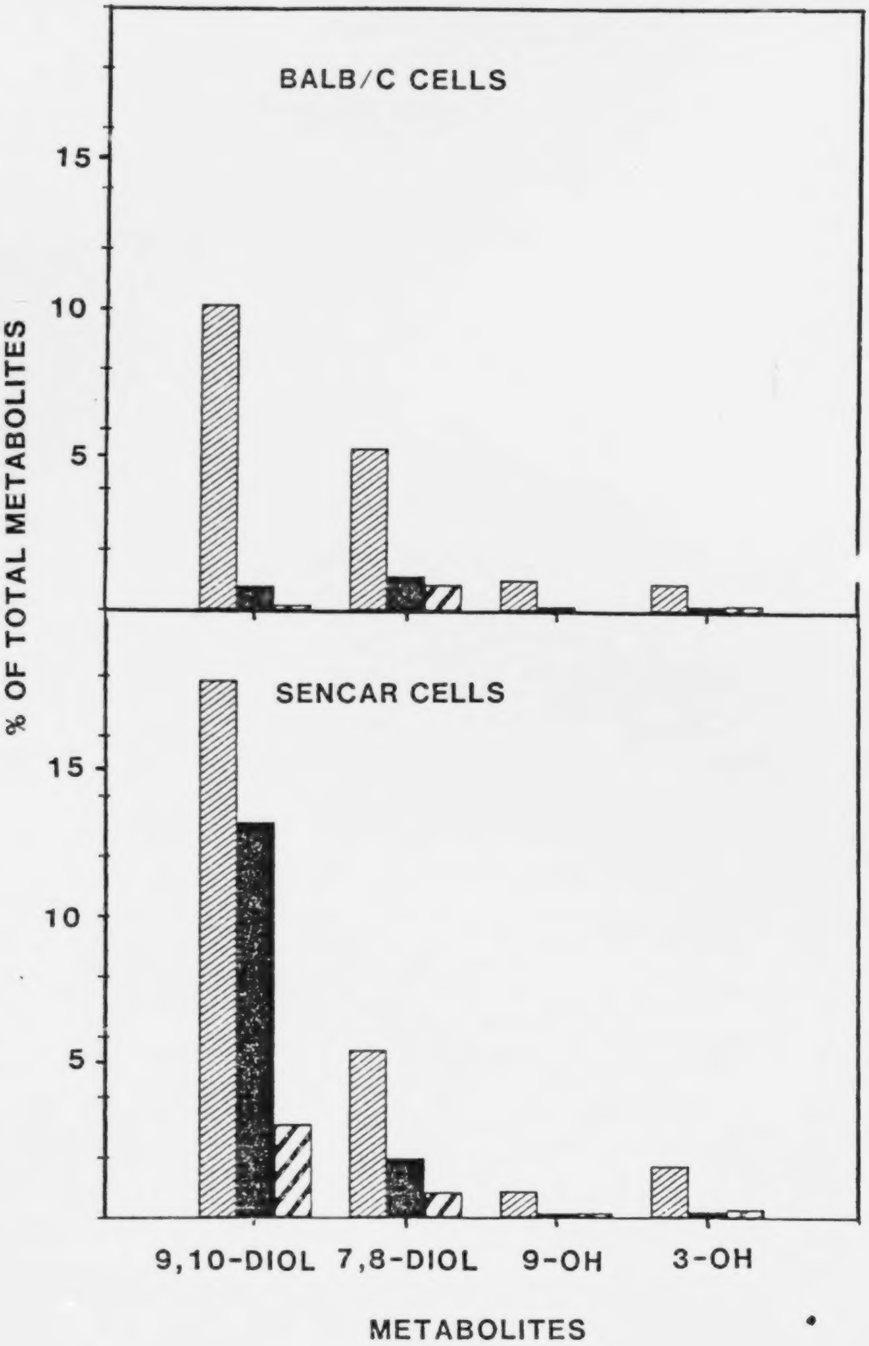

Figure 4. Percentage of individual B(a)P metabolites formed in BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. The metabolites were analyzed as described in the legend of Fig. 3. Exposure times were: ▨ 5 hours; ■ 24 hours; ▨ 48 hours.

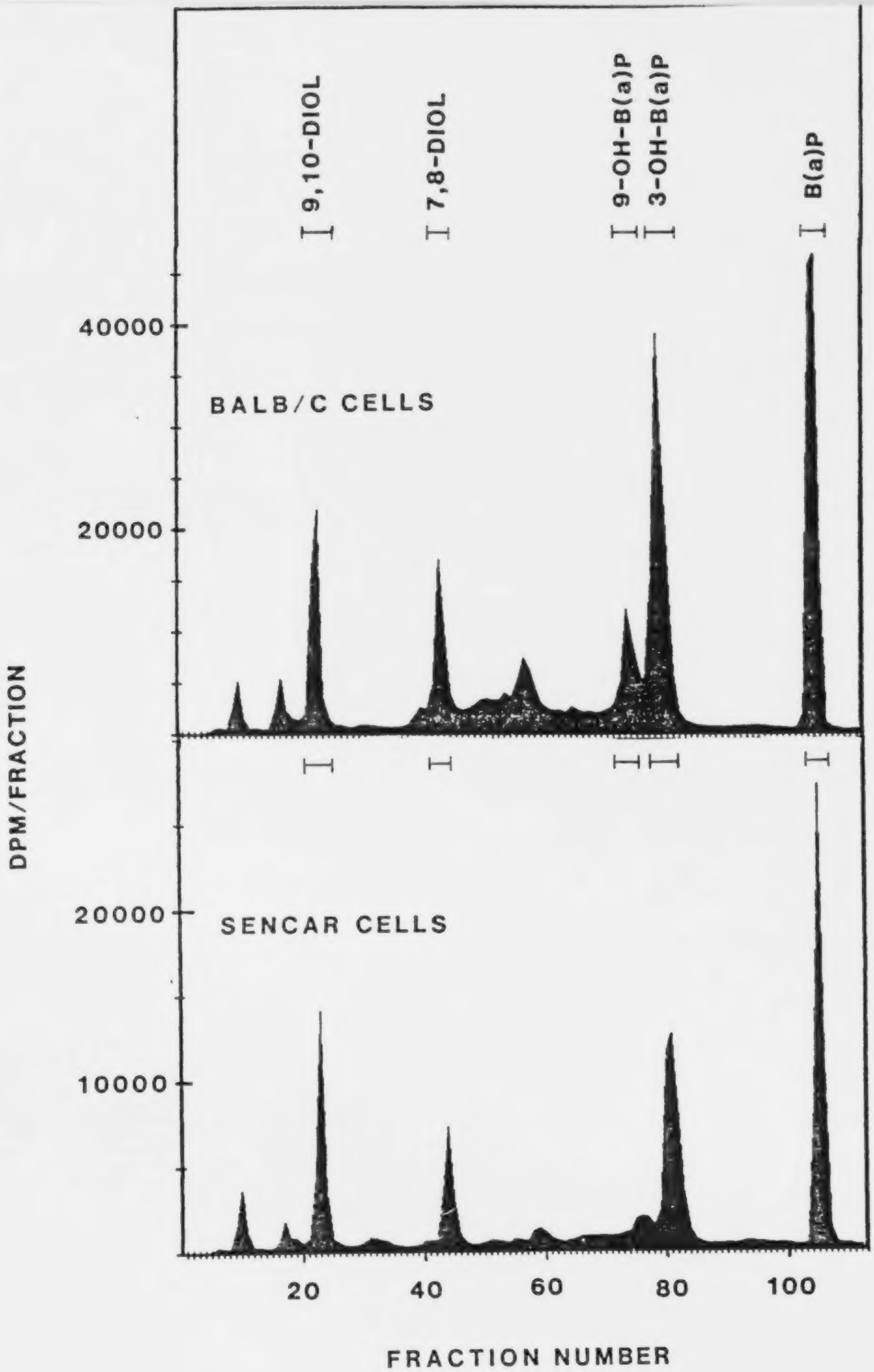

Figure 5. HPLC elution profile of the chloroform-extractable B(a)P metabolites present in β-glucuronidase treated medium from BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. The medium samples were treated with β-glucuronidase as described in Materials and Methods prior to extraction and analysis as described in the legend to Fig. 3. The elution positions of B(a)P metabolite markers are shown by the ⊢⊣ .

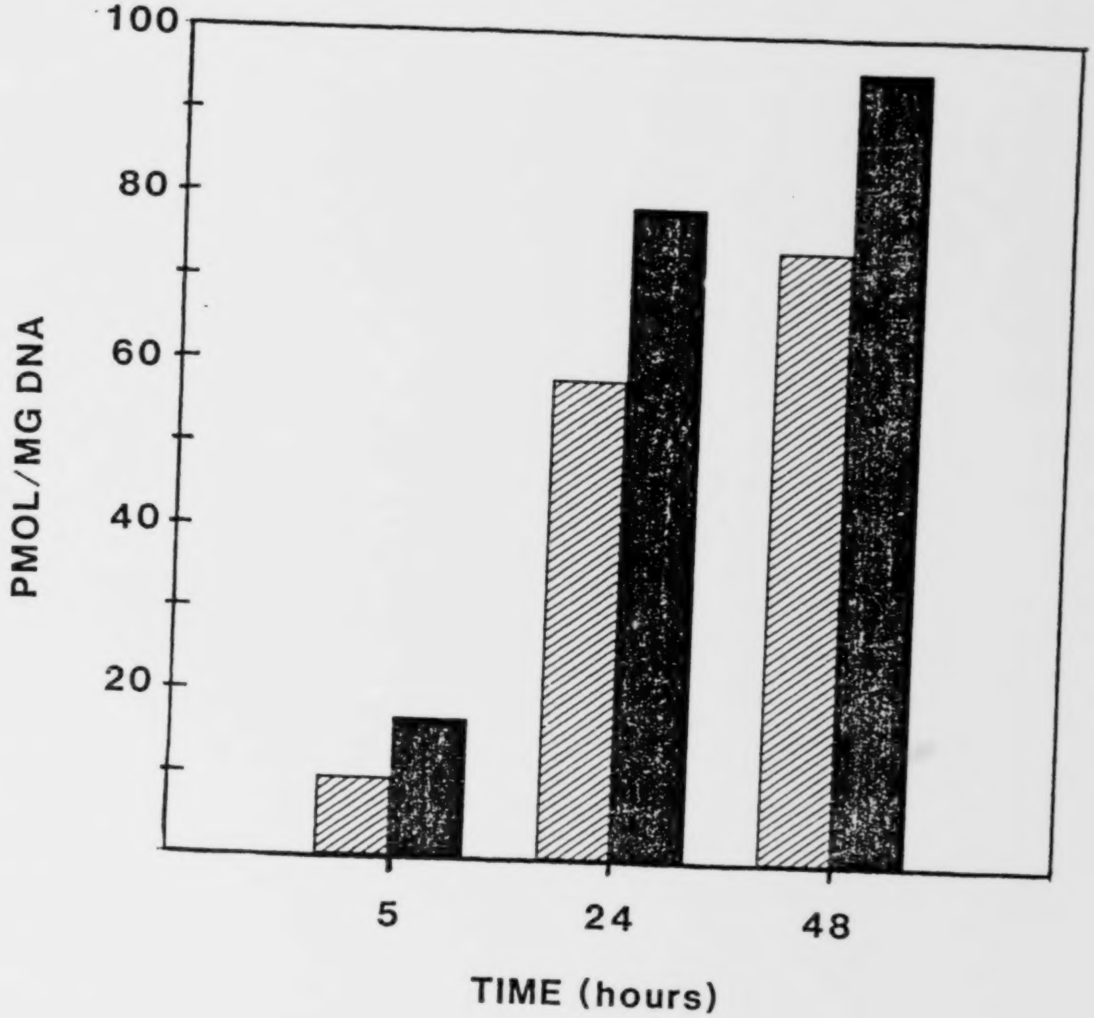

Figure 6. Total binding levels of B(a)P to DNA in BALB/c and SENCAR mouse embryo cell cultures. Cultures were exposed to [^{3}H]B(a)P for the times stated and the DNA was isolated and level of binding determined as described in Materials and Methods. ▨ BALB/c cell cultures. ■ SENCAR cell cultures.

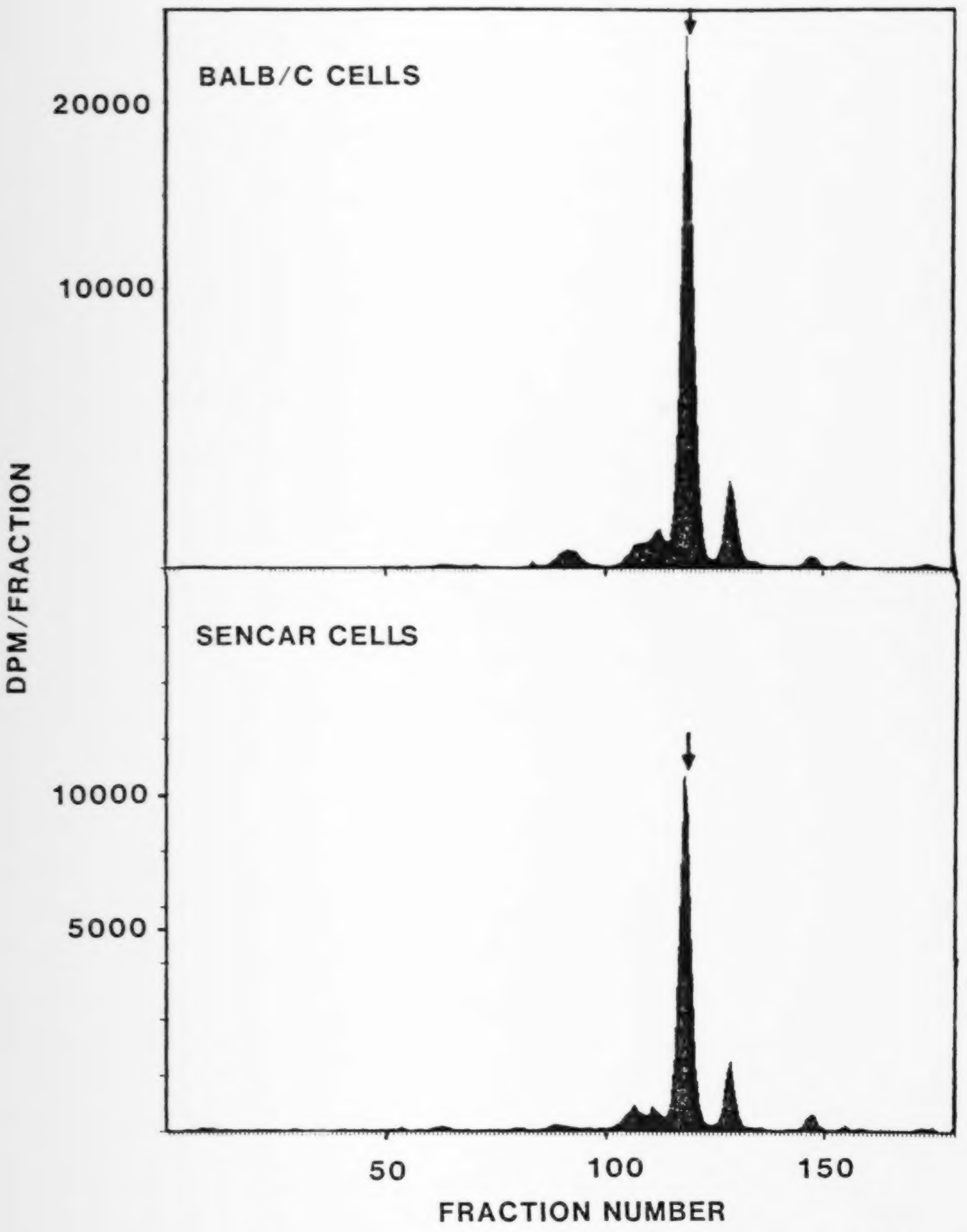

Figure 7. HPLC elution profile of B(a)P-DNA adducts formed in BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. Cultures were exposed to [^{3}H]B(a)P for 24 hours and the DNA was isolated, enzymatically degraded to deoxyribonucleosides and the B(a)P-deoxyribonucleoside adducts analyzed by HPLC as described in Materials and Methods. The elution position of a [^{14}C](+)anti-B(a)PDE-dG adduct that was added to each sample as a marker is shown by the arrow.

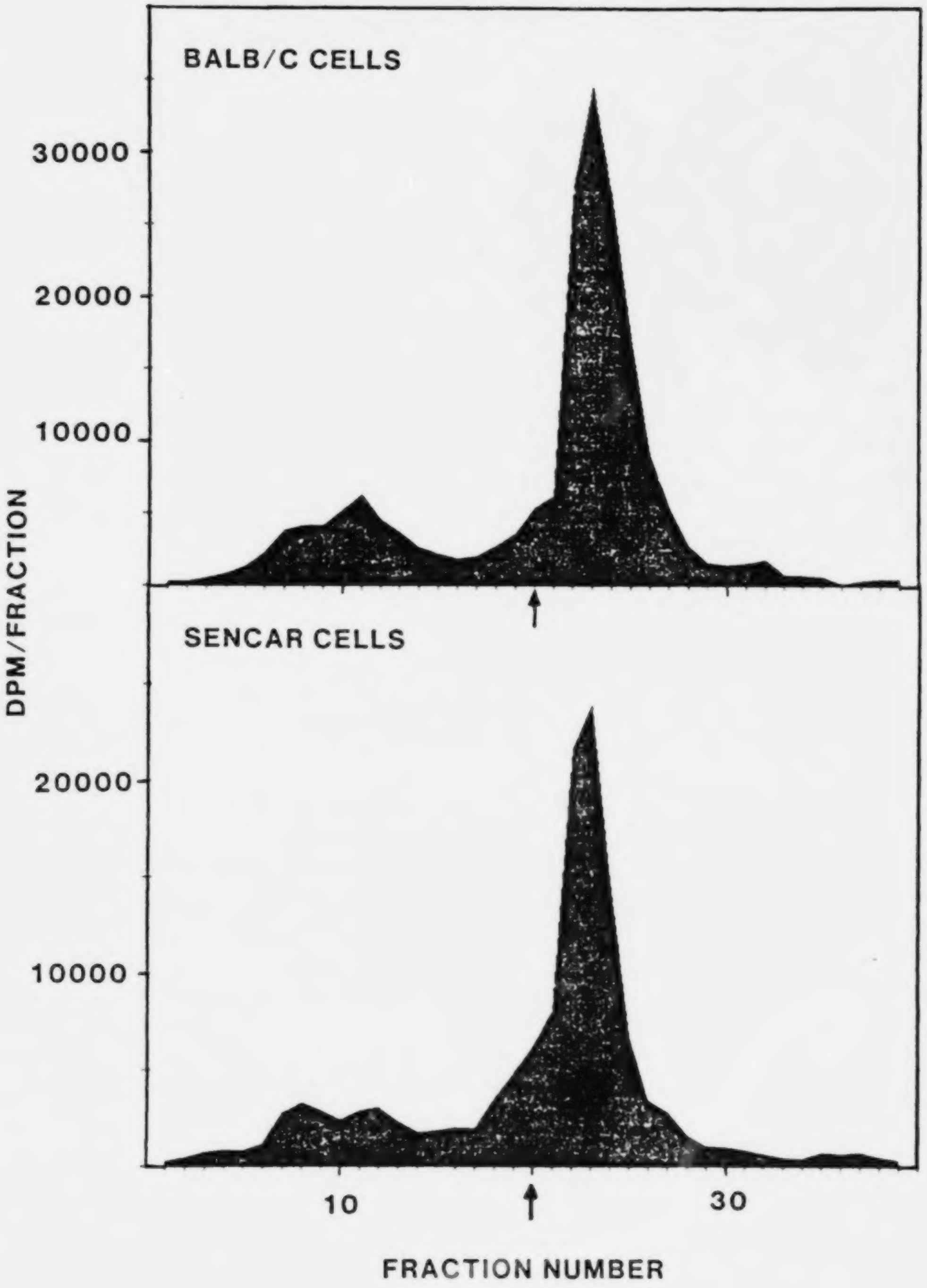

Figure 8. Immobilized boronate chromatography elution profile of B(a)P-DNA adducts from BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. Cultures were exposed to [^{3}H]B(a)P for 24 hours and the B(a)P-deoxyribonucleoside adducts were isolated and chromatographed as described in Materials and Methods. The arrow indicates the fraction number at which sorbitol was added to the eluting buffer.

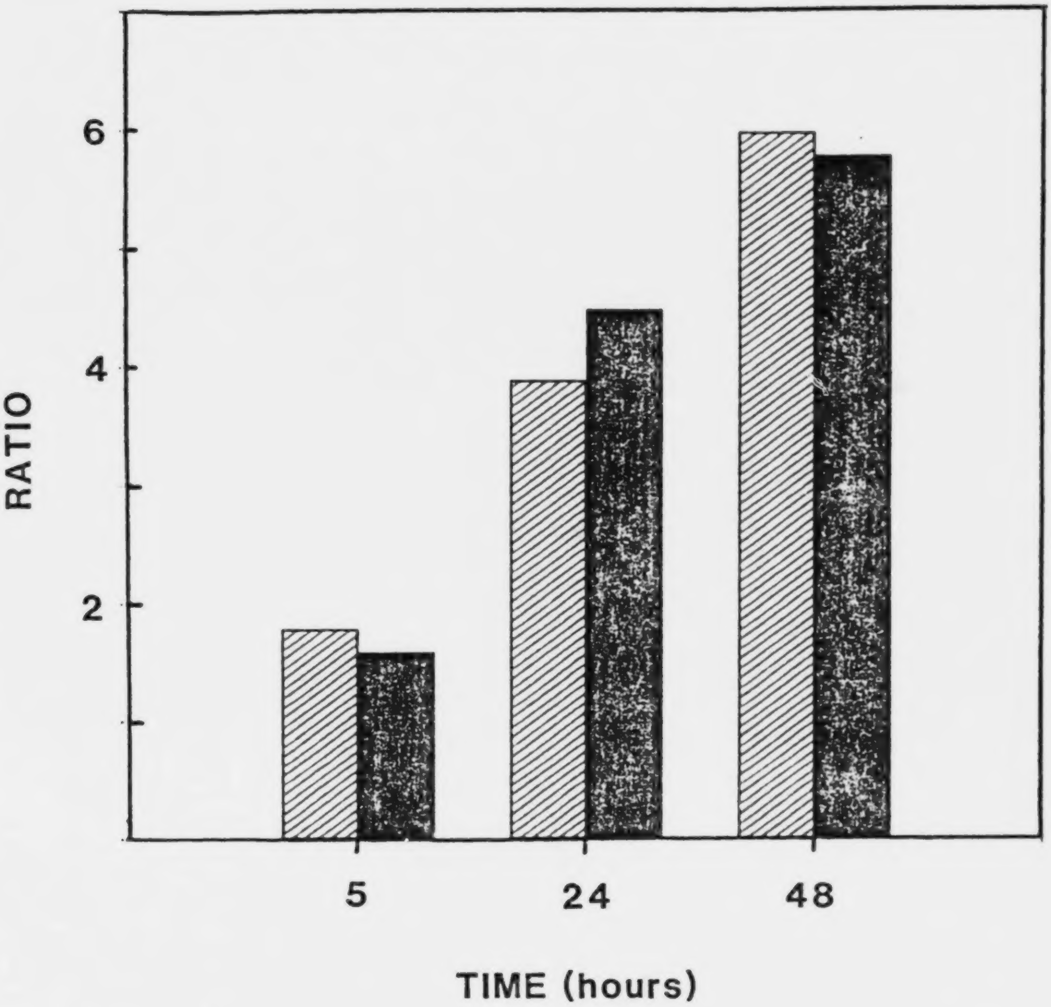

Figure 9. Ratio of anti to syn-B(a)PDE-DNA adducts present in BALB/c and SENCAR mouse embryo cell cultures. The cultures were treated and B(a)P-DNA adducts analyzed by immobilized boronate chromatography as described in the legend to Figure 8. The ratio of material eluted in the sorbitol buffer (anti-B(a)PDE-DNA adducts) to that eluted in the morpholine buffer (syn-B(a)PDE-DNA adducts) was calculated. ▨ BALB/c cell cultures. ■ SENCAR cell cultures.

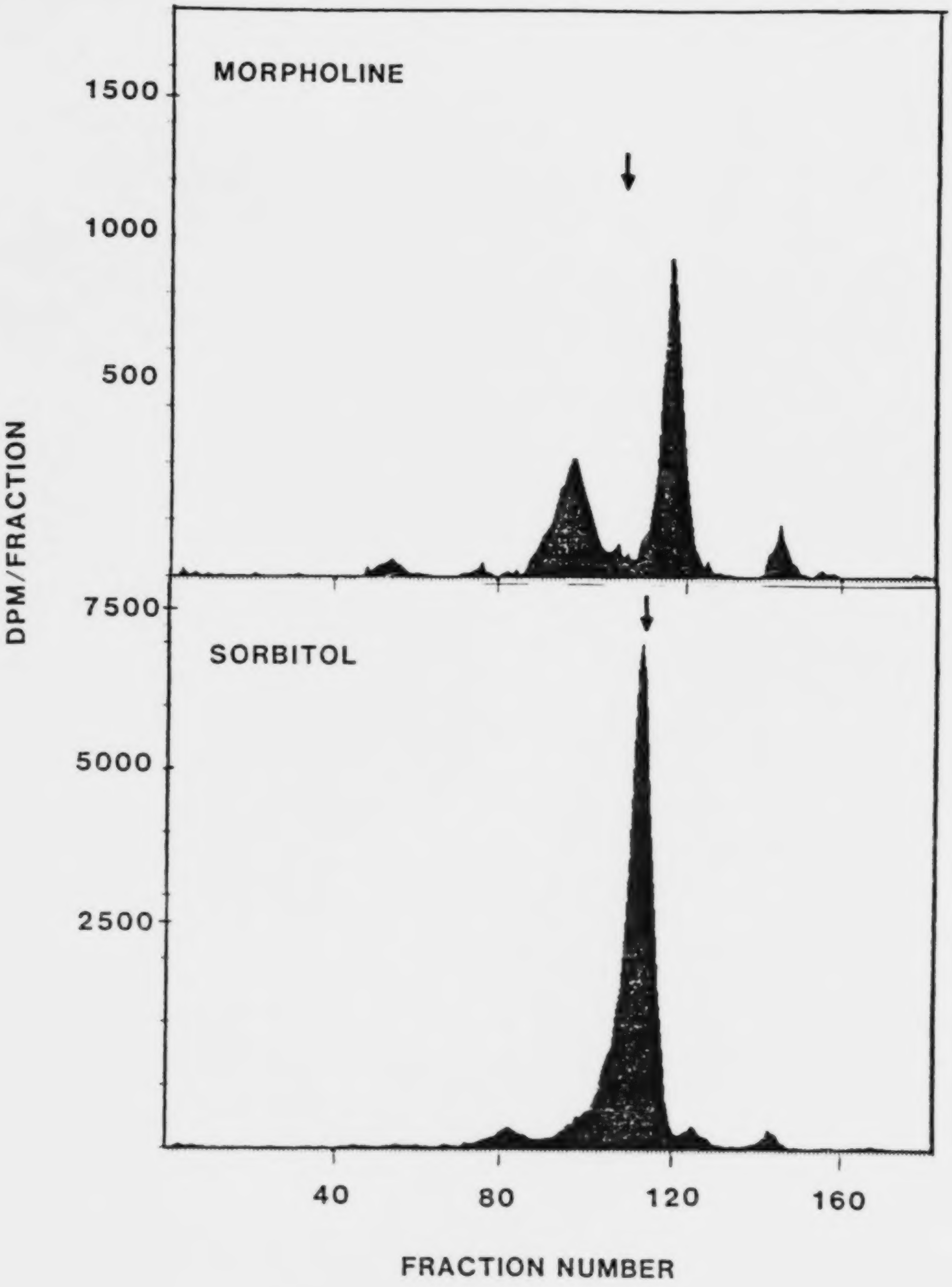

Figure 10. HPLC elution profile of the B(a)P-DNA adducts present in the morpholine (top) and sorbitol (bottom) fractions of the immobilized boronate column eluate of (Figure 8, top) DNA from B(a)P treated BALB/c mouse embryo cell cultures. The samples were analyzed by HPLC as described in Materials and Methods. The elution position of a [^{14}C](+)anti-B(a)PDE-dG adduct marker is shown by the arrow.

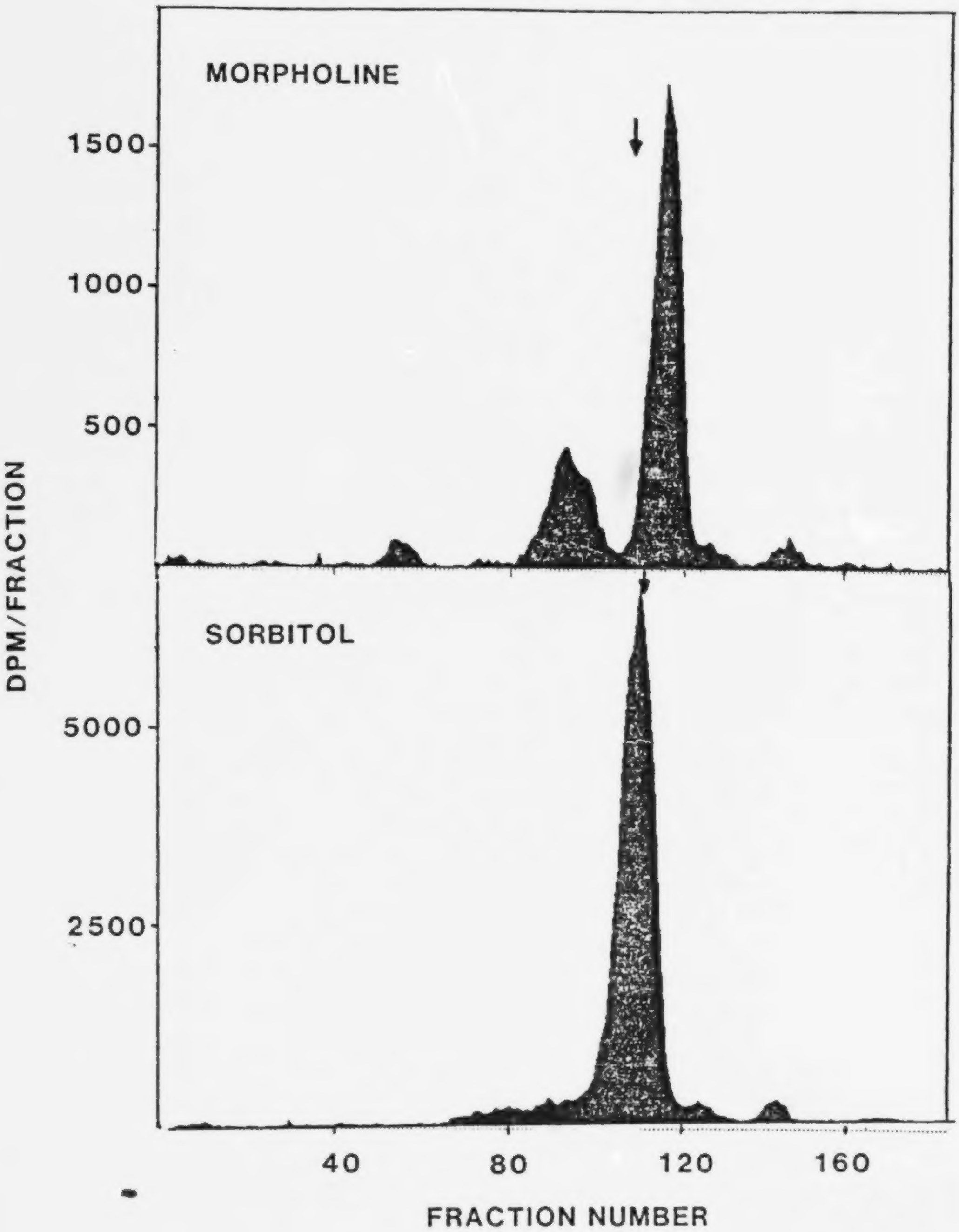

Figure 11. HPLC elution profile of the B(a)P-DNA adducts present in the morpholine (top) and sorbitol (bottom) fractions of the immobilized boronate column eluate (Figure 8, bottom) of DNA from B(a)P-treated SENCAR mouse embryo cell cultures. The samples were analyzed by HPLC as described in Materials and Methods. The elution position of a [^{14}C](+)anti-B(a)PDE-dG adduct marker is shown by the arrow.

BINDING AND DISTRIBUTION STUDIES IN THE SENCAR MOUSE OF COMPOUNDS DEMONSTRATING A ROUTE DEPENDENT EFFECT

by

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Key Words:

benzo(a)pyrene
urethane
ethyl carbamate
acrylamide
Sencar mouse
initiation-promotion assay
carcinogenesis

Previous investigators have determined that benzo(a)pyrene was much more effective in causing skin papillomas if applied topically than when administered orally in the initiation-promotion assay in Sencar mouse whereas urethane and acrylamide caused a higher percentage of mice with papillomas and more tumors per mouse when given orally. In an attempt to understand the reason for this discrepancy in route dependency, ^{3}H-benzo(a)pyrene, ^{14}C-urethane and ^{14}C-acrylamide were administered as single doses orally or topically to male Sencar mice. Distribution in skin, stomach, liver and lung was determined for time periods up to 48 hours. The binding of these compounds to DNA, RNA and protein in these tissues was determined 6 and 48 hours after administration. For all three compounds, high concentrations were found in the skin following topical application, but very little material reached this target organ following oral administration. In contrast, the internal organs generally contained more material after oral administration. The binding of labelled compound to DNA, RNA and protein generally reflected the distribution data with more compound being bound in the stomach, liver and lung after oral administration compared to topical application and the opposite being true for the skin. This was particularly evident for the benzo(a)pyrene. The results suggest that differences in distribution to the skin and binding to macromolecules following oral or topical administration cannot explain the greater tumorigenicity of urethane and acrylamide after oral administration in the Sencar mouse.

INTRODUCTION

The initiation-promotion assay has proven to be a promising assay for the determination of tumorigenicity. It has been refined for use with the Sencar mouse on the basis of the sensitivity of this strain (1). After application of the test compound to the skin followed by a promotion schedule, papilloma formation on the skin can be quantified. As more compounds have been tested using this system, differences in tumor incidence have been observed which are dependent upon the route of administration as well as the strain (2).

Bull *et al*. (3) found that both the percentage of mice developing papillomas and the number of papillomas per mouse was greater when benzo(a)pyrene was applied topically than when administered orally. In contrast these authors found that the administration of urethane (ethyl carbamate) orally resulted in a greater tumor incidence and a higher proportion of animals with tumors than when the material was applied topically. A similar finding was observed with acrylamide which demonstrated greater activity when the oral route of administration was employed (4). These results are summarized in Table 1 where the cumulative papilloma formation at the end of 52 weeks is considered.

The purpose of the present studies was to determine if the route differences observed with these three compounds could be explained by differences in distribution following administration via the oral and topical routes. A second hypothesis tested was that the difference in sensitivity was related to the binding of the compounds to macromolecules, principally DNA, rather than to simple distributional differences.

MATERIALS AND METHODS

Animals

Male Sencar mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were housed individually in stainless steel metabolism cages.

Treatment of animals.

(G-^{3}H)-Benzo(a)pyrene (Amersham Corporation, Arlington Heights, IL) was diluted with unlabelled benzo(a)pyrene (Sigma Chemical Co., St. Louis, MO). It was administered orally via intubation in an emulsion of 10% GAF Emulphor EL 620 and 90% water or was applied topically in acetone. The back of each animal was shaved 36 to 48 hours prior to treatment, and the compound was administered at a dose of 50 mg/kg and a specific activity of 630 uCi/mmol in the distribution studies and 25.2 mCi/mmol in the binding studies. Groups of at least 5 animals were sacrificed at 0.5, 1, 6, 12, 24 and 48 hours after treatment in the distribution studies and at 6 and 48 hours in the binding studies.

Ethyl-1-^{14}urethane (New England Nuclear, Boston, MA) was diluted with unlabelled urethane (Sigma Chemical Co.) and administered at a dose of 100 mg/kg (89 uCi/mmol) for the distribution studies and 62 mg/kg (3.59 mCi/mmol) for the binding studies. Water was the vehicle for the oral studies and acetone for the topical. Observations were made at similar times starting at one hour. 2,3-^{14}C-Acrylamide (New England Nuclear, Boston, MA for the distribution studies and Pathfinder Laboratories, St. Louis, MO for the binding studies) was diluted with unlabeled acrylamide (Bethesda Research Laboratories, Gaithersburg, MD) and administered at a dose of 100 mg/kg with a specific activity of 56.9 uCi/mmol for the distribution studies and

1.185 mCi/mmol for the binding studies. The compound was administered in water orally or in ethanol topically. Groups of mice were sacrificed starting at 15 minutes after administration of the compound.

Treatment of Tissues in Distribution Studies

Duplicate 50 to 100 mg portions of skin, liver, lung and stomach were obtained. In the acrylamide studies, testes were also removed. The tissues were minced and digested in 1.0 ml NCS tissue solubilizer (Amersham Corporation) for 24 hours at 50°C. After dissolution, 10 ml of Aquasol II (New England Nuclear) were added, and the samples were counted in a liquid scintillation spectrometer. ^{14}C-Toluene and ^{3}H-toluene were added as internal standards and the samples recounted.

Isolation and Analysis of Macromolecules

In the urethane studies, portions of the skin, stomach, liver and lungs were removed and homogenized in an aqueous solution (1% NaCl, 1% tri-isopropylnaphthalene sulfonic acid, 6% sec-butyl alcohol and 6% p-aminosalicylate). Skin was frozen in liquid nitrogen and shattered prior to homogenization. Tissues including the testes were similarly treated in the acrylamide studies. In the benzo(a)pyrene experiments the epidermal layers were removed by a thermal method (5) and homogenized with a Polytron PT-10.

The DNA, RNA and protein were isolated by a modification of the methods of Kirby and Cook (6) and Marmur (7). An equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase. After shaking and centrifugation, the aqueous layer containing the RNA and DNA was removed, washed with the same solution and recentrifuged. The DNA was precipitated with ethoxyethanol and the RNA with ethanol and cooling. The epidermal samples were treated with protease K. The samples were solubilized in NCS solution, and Aquasol II was added. They were counted as in the distribution studies. Protein from the interface was quantified using the Lowry procedure (8).

Data Expression and Analysis

Since no attempt was made to differentiate between parent compound and metabolites or to identify the labelled material bound, data are expressed as equivalents. Differences between the two routes were analyzed using Student's t-test at the individual time points. A p value of 0.05 was selected to represent statistical significance.

RESULTS

When benzo(a)pyrene was administered topically, the concentration in the skin peaked at one hour (Fig. 1). There was a rapid decline by six hours, and label was slowly lost from the skin after that time. Very little material reached the skin when it was administered orally. In contrast, only small amounts of benzo(a)pyrene were in the stomach tissue when the compound was given topically compared to the material found soon after oral administration although little difference was observed between the two routes after 12 hours. Similar findings were observed in the liver and lung.

More benzo(a)pyrene was bound to the DNA of the liver, lung and stomach in the orally treated animals than in the topically dosed animals at both 6 and 48 hours (Fig. 2). However, the amount of label bound to DNA after topical application was greater in the skin (epidermis) than in other tissues and was much higher than that observed after oral administration. Similar differences were exhibited in RNA and protein.

When urethane was applied to the backs of the Sencar mice, the concentrations found in the skin were many times higher than those found after oral administration (Fig. 3). The decline with time was quite slow. In contrast, the concentration of labelled material found in the stomach tissue was quite low after topical application and was less than that seen after oral administration at all time points. The liver and lungs displayed a similar pattern and level as did the stomach suggesting a fairly even distribution among these internal organs.

When the binding of the ^{14}C label to DNA was measured, the differences between the routes of administration were not as great as those observed in the distribution studies (Fig. 4). No significant differences were observed at the six hour time point. However at 48 hours, more label was bound to DNA in the stomach and liver of the orally treated animals and in the skin of the topically dosed animals. The pattern of binding to RNA was quite similar except that there was no difference in the skin between the two routes. The binding of urethane to protein was greater in the stomach 6 hours after oral administration and in the skin 6 and 48 hours after topical application.

Although high concentrations of ^{14}C-acrylamide were present in the skin following topical application, very little was present in this tissue after oral administration (Fig. 5). Surprisingly high concentrations were observed in the stomach after topical application, but these decreased rapidly and from 6 to 24 hours were lower than those measured in the orally dosed animals. In liver, lung and testes, concentrations peaked 30 to 60 minutes after dosing by either route and then sharply declined. In general the oral dosing resulted in higher concentrations of acrylamide in the tissues through 24 hours, but by 48 hours there were no differences between the groups.

The distribution data were reflected in the binding of acrylamide to macromolecules (Fig. 6). Binding to DNA, RNA and protein in the internal organs was elevated in the orally treated mice compared to the topically treated animals whereas the opposite was true for the skin.

DISCUSSION

Benzo(a)pyrene is a well established carcinogen. While much less potent, urethane has been demonstrated to cause tumors in the lung (9,10), liver (11, 12), stomach (13) and skin (14, 15). Acrylamide causes skin papillomas in the Sencar mouse and lung adenomas in the A/J mouse (4) and is structurally related to vinyl carbamate hypothesized as the active metabolite of urethane (16).

The purpose of the current studies was to attempt to explain the differences in the incidence of skin papillomas in Sencar mice where Bull *et al*. (3, 4 and personal communication) found that benzo(a)pyrene caused many more tumors when administered topically whereas both urethane and acrylamide were more tumorigenic by the oral route (Table 1). One possibility was that urethane and acrylamide, which are much more hydrophilic than benzo(a)pyrene, did not penetrate the skin well and higher concentrations were achieved in this tissue after oral administration due to delivery to this tissue via the blood. The data, however, indicated that this did not occur. These two compounds resembled benzo(a)pyrene in that higher concentrations of labelled material were achieved in the skin after topical application than after oral dosing.

A second possibility that the urethane and acrylamide were activated by the liver and/or other organs to proximate carcinogens which were subsequently transported to the target organ, skin, was examined by determining the degree of binding to macromolecules. This does not appear to be the case. Although the differences were not as striking as with benzo(a)pyrene, in general the binding of the urethane and acrylamide to DNA, RNA and protein was greater in the internal organs following oral administration and in the skin following topical application. Thus neither the distribution nor the binding data can adequately explain the route differences in the induction of papillomas in Sencar mice. Further studies are necessary to evaluate a third possibility that there are differences in the DNA adducts formed following the administration of the compounds by the two routes.

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Acknowledgments

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Table 1. Tumors in Sencar mouse skin following oral and topical administration.[a]

Compound	Route	Dose mg/kg	% Animals with tumors	Tumor per Animal
Benzo(a)pyrene	oral	10	20	0.20
		30	4	0.04
		100	24	0.24
	topical	0.01	20	0.28
		0.10	56	1.16
		1.00	92	13.24
Urethane	oral	30	24	0.24
		100	28	0.44
		300	68	1.20
	topical	30	16	0.16
		100	24	0.28
		300	24	0.36
Acrylamide[b]	oral	75	30	0.33
		150	58	1.00
		300	75	1.30
	topical	75	10	0.20
		150	28	0.33
		300	45	0.55

[a] Data are from Bull and Robinson (ref. 3 and 4 and personal communication).

[b] Divided into 6 applications over a two week period.

FIGURE 1. Distribution of ^{3}H-benzo(a)pyrene in tissues of Sencar mice following oral or topical administration. ^{3}H-Benzo(a)pyrene (50 mg/kg, 630 umols/mmol) was administered orally in an emulsion (open circles) or topically in acetone (solid squares). Values are mean ± SE for skin (A), stomach (B), liver (C), and lung (D). Asterisks denote significant differences between routes at the same time point ($p < 0.05$).

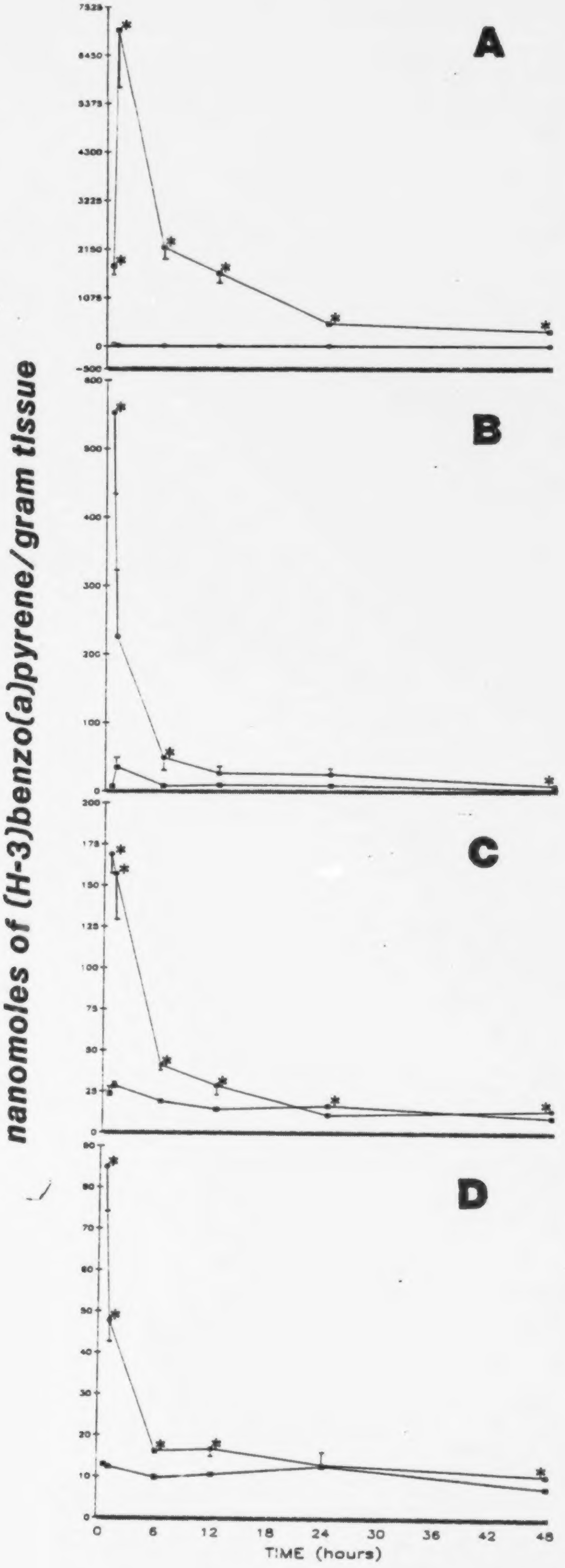

nanomoles of (H-3)benzo(a)pyrene/gram tissue
A
7525
6450
5375
4300
3225
2150
1075
0
-500
B
600
500
400
300
200
100
0
C
200
175
150
125
100
75
50
25
0
D
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
36
42
48
TIME (hours)

FIGURE 2. Binding of ^{3}H-benzo(a)pyrene to DNA, RNA and protein in tissues of Sencar mice 6 and 48 hours following oral or topical administration. ^{3}H-Benzo(a)pyrene (50 mg/kg, 25.2 mCi/mmol) was administered orally in an emulsion (hatched bars) or topically in acetone (solid bars). Values are mean ± SE. Asterisks denote significant differences between routes at the same time point for the same tissue ($p < 0.05$).

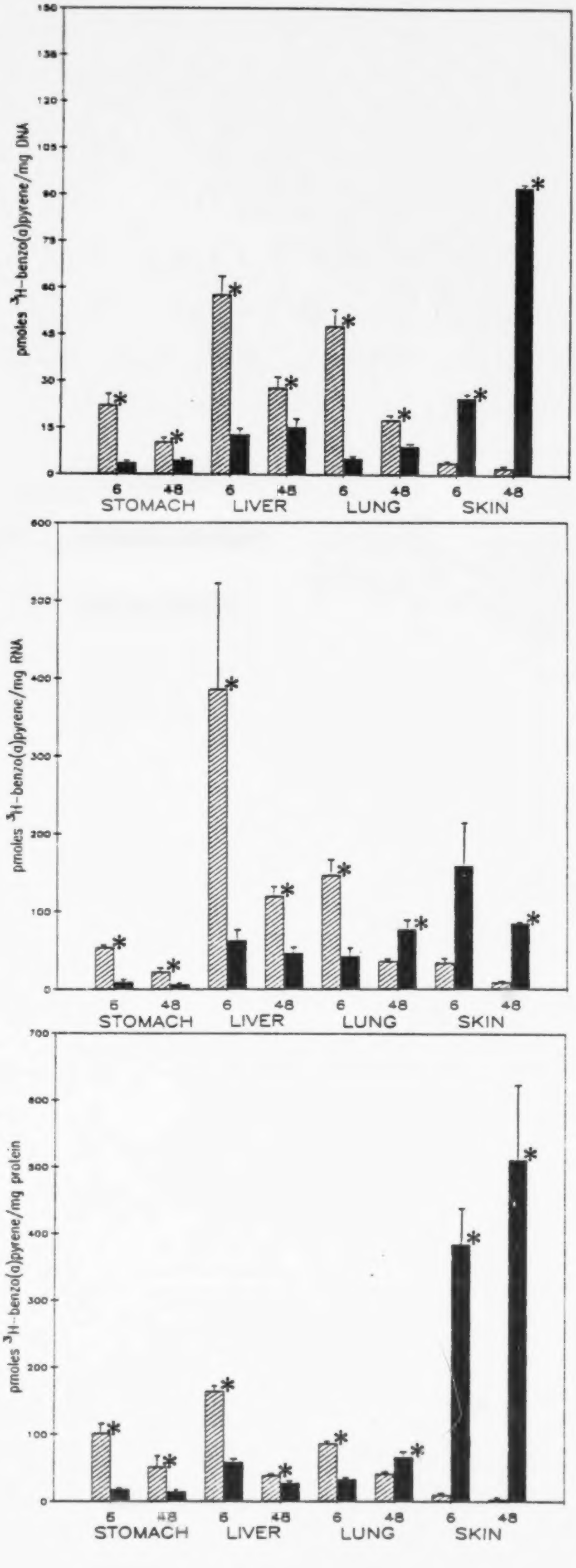

pmoles ³H-benzo(a)pyrene/mg DNA
0
15
30
45
60
75
90
105
120
135
150
*
6
48
STOMACH
LIVER
LUNG
SKIN
pmoles ³H-benzo(a)pyrene/mg RNA
0
100
200
300
400
500
600
pmoles ³H-benzo(a)pyrene/mg protein
0
100
200
300
400
500
600
700

FIGURE 3. Distribution of ^{14}C-urethane in tissues of Sencar mice following oral or topical administration. ^{14}C-Urethane (100 mg/kg, 89 uCi/mmol) was administered orally in water (open circles) or topically in acetone (solid squares). Values are mean ± SE for skin (A), stomach (B), liver (C) and lung (D). Asterisks denote significant differences between routes at the same time point ($p < 0.05$).

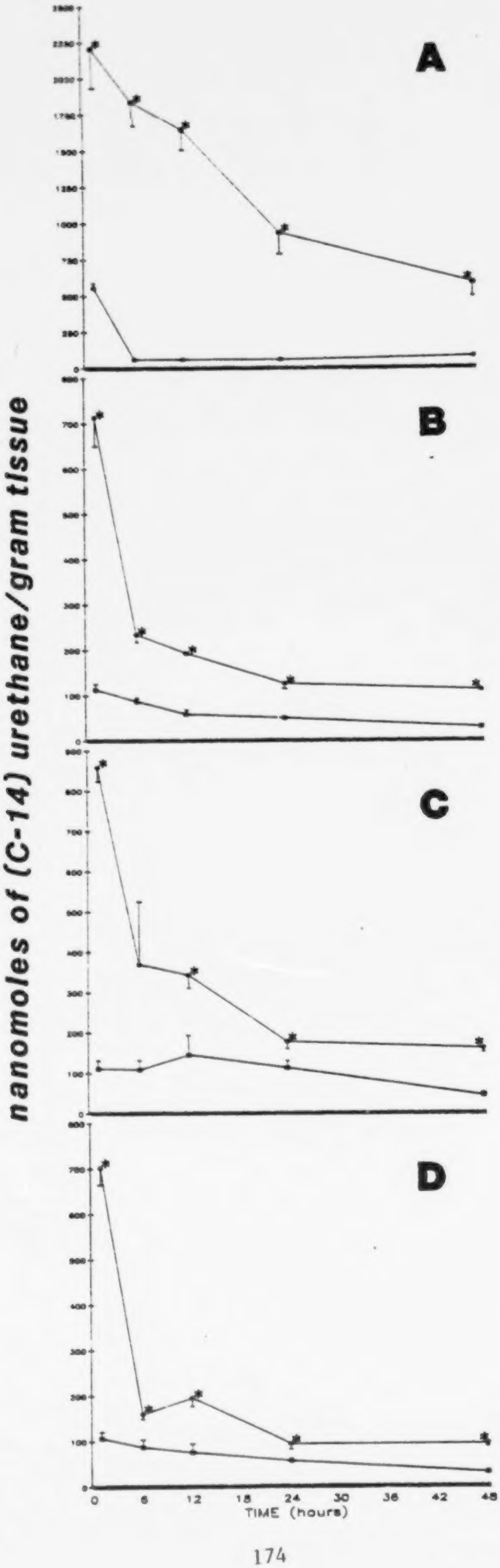
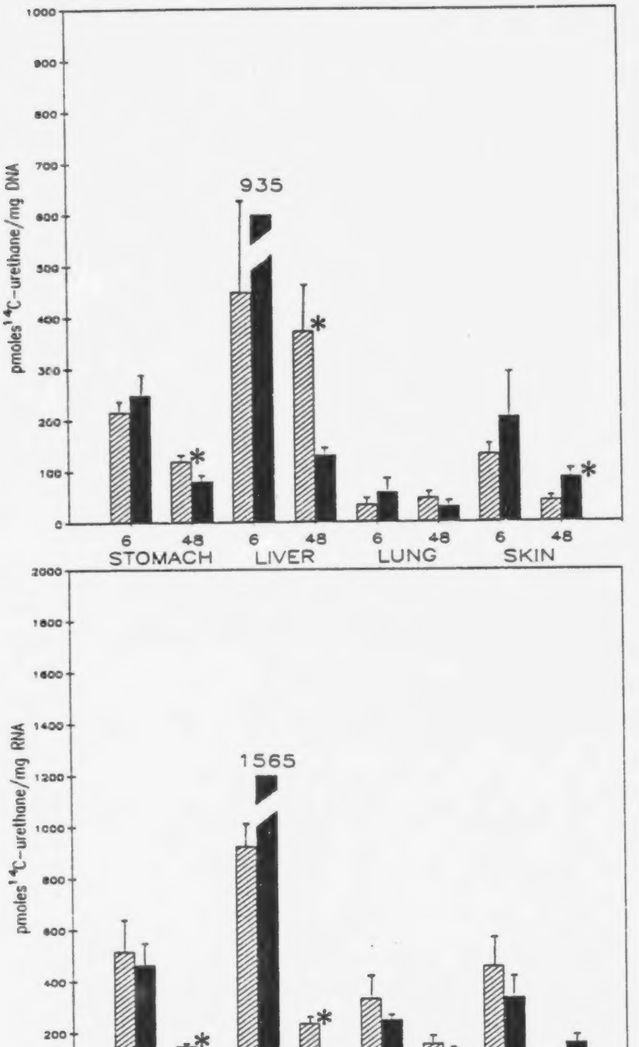

A
B
C
D
nanomoles of (C-14) urethane/gram tissue
TIME (hours)
0
6
12
18
24
30
36
42
48
0
250
500
750
1000
1250
1500
1750
2000
2250
2500
100
200
300
400
500
600
700
800
900

FIGURE 4. Binding of ^{14}C-urethane to DNA, RNA and protein in tissues of Sencar mice 6 and 48 hours after oral or topical administration. ^{14}C-Urethane (62 mg/kg, 3.59 mCi/mmol) was administered orally in water (hatched bars) or topically in acetone (solid bars). Values are mean ± SE. Asterisks denote significant differences between routes at the same time point for the same tissue ($p < 0.05$).



pmoles ¹⁴C-urethane/mg DNA
0
100
200
300
400
500
600
700
800
900
1000
935
*
*
*
6
48
6
48
6
48
6
48
STOMACH
LIVER
LUNG
SKIN
pmoles ¹⁴C-urethane/mg RNA
0
200
400
600
800
1000
1200
1400
1600
1800
2000
1565
*
*
6
48
6
48
6
48
6
48
STOMACH
LIVER
LUNG
SKIN
pmoles ¹⁴C-urethane/mg protein
0
130
260
390
520
650
780
910
1040
1170
1300
1486
*
*
*
6
48
6
48
6
48
6
48
STOMACH
LIVER
LUNG
SKIN

FIGURE 5. Distribution of ^{14}C-acrylamide in tissues of Sencar mice following oral or topical administration. ^{14}C-Acrylamide (100 mg/kg, 56.9 uCi/mmol) was administered orally in water (open circles) or topically in ethanol (solid squares). Values are mean ± SE for skin (A), stomach (B), liver (C), lung(D) and testes (E). Asterisks denote significant differences between routes at the same time point ($p < 0.05$).

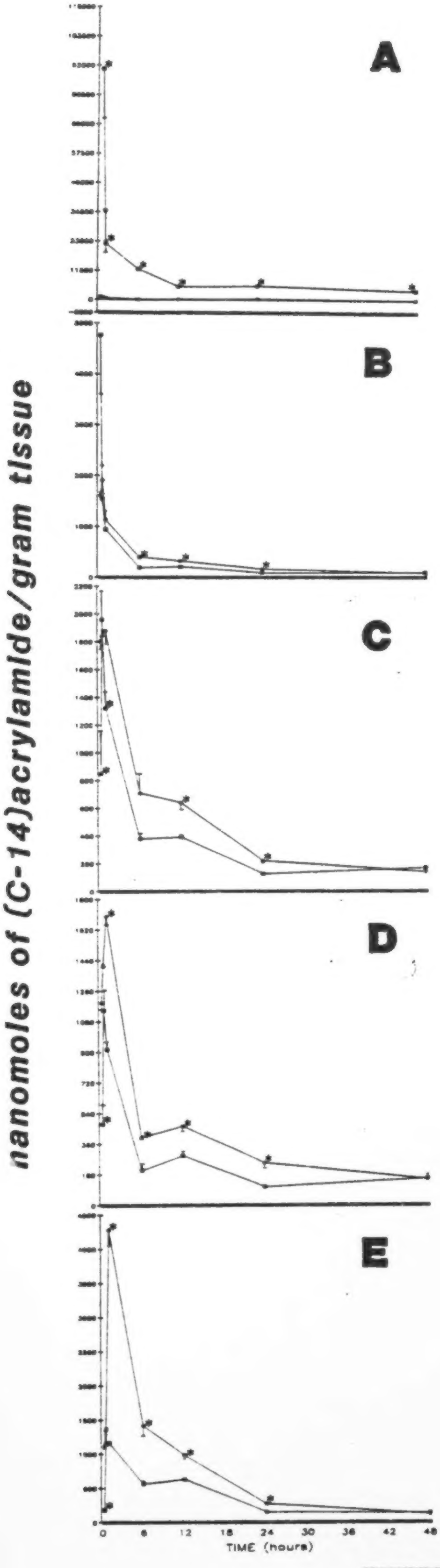


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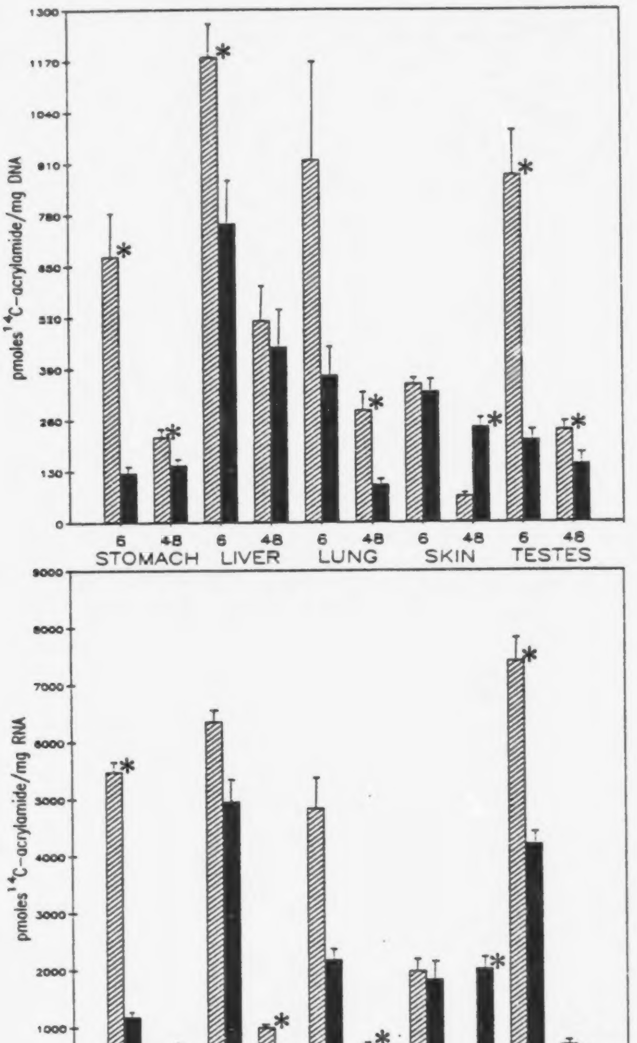
FIGURE 6. Binding of ^{14}C-acrylamide to DNA, RNA and protein in tissues of Sencar mice 6 and 48 hours after oral or topical administration. ^{14}C-Acrylamide (100 mg/kg, 1.185 mCi/mmol) was administered orally in water (hatched bars) or topically in ethanol (solid bars). Values are mean ± SE. Asterisks denote significant differences between routes at the same time point for the same tissue ($p < 0.05$).


pmoles ¹⁴C-acrylamide/mg DNA
0
130
260
390
520
650
780
910
1040
1170
1300
*
6
48
6
48
6
48
6
48
6
48
STOMACH
LIVER
LUNG
SKIN
TESTES
pmoles ¹⁴C-acrylamide/mg RNA
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
6
48
6
48
6
48
6
48
6
48
STOMACH
LIVER
LUNG
SKIN
TESTES
pmoles ¹⁴C-acrylamide/mg protein
0
575
1150
1725
2300
2875
3450
4025
4600
6
48
6
48
6
48
6
48
6
48
STOMACH
LIVER
LUNG
SKIN
TESTES

DIFFERENTIAL CARCINOGENIC EFFECTS TO SKIN AND INTERNAL TISSUES BY INTRAPERITONEAL INITIATION WITH 7,12-DIMETHYLBENZ[A]ANTHRACENE OR URETHANE AND TOPICAL 12-O-TETRADECANOYLPHORBOL-13-ACETATE PROMOTION IN FEMALE SENCAR AND BALB/c MICE

by

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Key words: SENCAR mouse, DMBA, urethane, TPA, tumor promotion, lung tumors, skin carcinogenesis, pulmonary alveolar type II tumors.

Abstract

Groups of female SENCAR or BALB/c mice were initiated once intraperitoneally with 300 μg of 7,12-dimethylbenz[a]anthracene (DMBA) or 20 mg of urethane at 7 weeks of age. One week later, mice received topically applied acetone or 12-O-tetradecanoylphorbol-13-acetate (TPA), once weekly, at 2.5 μg for weeks 1-6 and 1.25 μg for weeks 7-52. The skin lesions were evaluated clinically. A complete necropsy was performed on all mice at week 52. SENCAR mice exposed to DMBA/TPA and urethane/TPA had more skin tumors than SENCAR mice exposed to DMBA or urethane alone and to all experimental BALB/c mice. For DMBA/TPA-exposed mice, more than 3 times the number of skin carcinomas were observed histologically than clinically, most arising within papillomas. BALB/c mice developed more vascular and uterine tumors than did SENCAR mice injected with DMBA and more lung, and vascular tumors than SENCAR mice injected with urethane. TPA had no significant effect on internal tumor development in either SENCAR or BALB/c mice.

Introduction

SENCAR mice were bred for susceptibility to 2-stage carcinogenesis in mouse skin (1). It was demonstrated that these mice are many times more sensitive to skin carcinogenesis and tumor promotion by chemicals (2-4) and ultraviolet irradiation (5). Although inherent susceptibility to skin carcinogenesis was apparently not to be related to the ability to metabolize carcinogens, it was shown that SENCAR mice have a population of spontaneously initiated cells (4), as well as dark cells (3), possible targets of carcinogens. In an earlier study on 2-stage carcinogenesis in SENCAR and BALB/c mice, it was shown that SENCAR mice are considerably more sensitive than BALB/c mice (3). It was the aim of this study to evaluate the differential carcinogenic effects of these carcinogens to internal tumor development in female SENCAR and BALB/c mice and to observe the effects of a skin tumor promoter on these effects.

Materials and Methods

Animals

Two hundred female SENCAR mice (Oak Ridge National Laboratories, Oak Ridge, TN) and 200 female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were received and held until they were 7 weeks of age, and initiated at that time. All animals were housed 5 per 7" x 11 1/2" polycarbonate cage on 1/8" corn cob bedding according to strain and treatment. Water and Purina Lab Chow were available ad lib throughout the experiment. All water bottles and cages were changed and sanitized twice per week, the adequacy of food and water checked daily, and death checks performed twice daily.

Chemicals

Initiators. 7-12-Dimethylbenz[a]anthracene (DMBA) was purchased from Eastman Organic (Rochester, NY) and prepared as a solution of 1200 μg/ml in DMSO/steroid suspending vehicle (SSV) (10%/90%). The DMBA was first dissolved in the appropriate volume of DMSO (Pierce Chemicals, Rockford, IL) and then diluted with 9x the volume of SSV (NCI, Cancer Chemotherpay Branch). Working solutions were prepared under yellow light in the carcinogen handling room hood, contained in amber multidose serum vials, and administered immediately to the animals. Stocks of DMBA used for dose preparations were stored at -20°C over drierite in amber glass vials.

Urethane purchased from Matheson, Coleman and Bell (Norwood, OH) was prepared as a solution of 80 mg/ml in sterile saline. Working solutions were prepared in amber multidose serum vials under yellow light in the hood of the chemical carcinogen handling room. Solutions were prepared immediately prior to dosing. Stocks of urethane used for dose preparations were stored at room temperature in foil wrapped vessels.

Promoter. 12-0-Tetradecanoylphorbol-13-acetate (TPA) purchased from CCR, Inc. (Eden Prairie, MN) was prepared as a 12.5 μg/ml or 6.25 μg/ml solution in acetone (Baker Chemical, Reagent Grade) depending on the dose per mouse per treatment given. Stock solutions of TPA were kept in tightly stoppered glass vessels which were wrapped in foil and kept refrigerated between uses. Undissolved stocks of TPA were stored at -20°C over drierite and in the dark.

Preparation of Animals and Methods of Administration

Mice receiving single intraperitoneal (i.p.) administration of DMBA (300 μg) or urethane (20 mg) as initiator were so treated at 0 experimental time (7 weeks of age). DMBA (1200 μg/ml in DMSO/SSV), urethane (80 mg/ml in saline), or the appropriate vehicle was dosed under a portable charcoal-filtered lab hood by the injection of 0.25 ml/mouse from 1 ml tuberculin syringes attached to appropriate sized hyperdermic needles.

TPA solutions (12.5 μg/ml or 6.25 μg/ml in acetone) or acetone vehicle were applied to appropriate groups once weekly from the first week after initiation through the 52nd experimental week. TPA or acetone solutions were applied topically at 0.2 ml/week. All mice were shaved biweekly at least 24 hours prior to promotion, in a consistent and standardized area to expose the back skin to chemical treatments applied topically. Solutions were applied from Eppendorf multipipettes delivering 0.2 ml of solution. Disposable tips were changed between groups receiving TPA or vehicles. The concentration of stocks of TPA used from week 1-6 was 12.5 μg/ml and 6.25 µg/ml from week 7-52. This resulted in doses of 2.5 µg/mouse or 1.25 µg/mouse per week, respectively. This change was undertaken when initial toxic skin reactions to TPA, which had previously been shown to be progressive, were observed.

Experimental Design

Table 1 summarizes the group designation, composition, and treatment for each group within this study. Mice were weighed monthly and biweekly papilloma counts were performed as previously described (3).

Pathology

Necropsies were performed on all animals found dead, moribund mice sacrificed during the study, or sacrificed at termination (52 weeks) unless precluded by autolysis or cannibalization. The following tissues were routinely preserved in 10% formalin: skin (all malignant and benign tumors, any TPA induced skin lesions and the routine collection of backskins from all animals), spleen and pancreas, grossly abnormal lymph nodes, and other lesions, lungs, heart, thymus, trachea, larynx and thyroid, lung tumors, kidneys and adrenals, liver, ovaries and uterus, brain and pituitary. Avidin-biotin immunocytochemistry was performed to localize keratin in trypsinized selected skin tumors (6) using rabbit antibodies to human keratin (DAKO Corp., Santa Barbara, CA) and to localize surfactant apoprotein (SAP) and Clara cell antigen (CCA) in lung tumors (6) and the Vectastain kit (Vector Laboratories, Burlingame, CA).

Results

Body Weight and Survival

The growth of female SENCAR mice in all groups was similar except for mice exposed to DMBA and TPA. The latter mice gained weight at a considerably slower rate than did other SENCAR mice (Fig. 1). BALB/c mice of all groups gained weight at the same rate (Fig. 1).

SENCAR mice exposed to DMBA and TPA also had the worst survival rate (Fig. 2) since only 23% of the mice survived to the end of the experiment while 63% of the SENCAR mice receiving urethane and TPA were alive and 77-100% of the mice in other groups survived. From 87-100% of the BALB/c mice were alive at 52 weeks (Fig. 2).

Tumors in Control Mice

Fifty-two weeks after treatment with the initiator vehicle (DMSO/SSV or acetone) and promoter (TPA) or promoter vehicle (acetone), at 59 weeks of age, skin and other tumors were found in SENCAR or BALB/c mice (Tables 2 and 3). TPA caused many skin tumors in SENCAR mice after DMSO/SSV vehicle initiation, but very few after saline vehicle initiation.

DMBA Initiation and TPA Promotion

SENCAR mice dying early after receiving DMBA and TPA had many skin tumors as previously reported (3) (Table 2). The occurrence of skin (Fig. 3), vascular, and uterine tumors was strain-related at time of necropsy (Table 2). By the end of the experiment in the DMBA/TPA SENCAR mice, there were 17 mice with 37 skin squamous cell carcinomas (10 seen grossly), 3 of which metastasized to lymph nodes and 2 to lungs. Several carcinomas were seen to arise within papillomas (Fig. 4). Keratin was found in well and poorly differentiated carcinomas (Fig. 5). The vascular tumors were usually small hemangiomas and were found in 35 of 60 BALB/c mice and 7 of 60 SENCAR mice exposed to DMBA. In BALB/c mice, 57 vascular tumors were found in 35 mice, usually on serosal surfaces of intestines (18 mice), uterus (23), mesentery (5), and other abdominal tissues (11). Uterine polypoid adenomas were found more frequently in BALB/c mice (11/60) than in SENCAR mice (1/60). Other tumors were found in similar incidences in both strains, although mammary and intestinal tumors were found only in SENCAR mice. Control mice had few tumors of these types. Lung tumors (adenomas or adenocarcinomas) occurred in higher histological incidence (14/30 vs. 10/30) or gross incidence (17/30 vs. 11/30) and greater gross multiplicity (2.0 vs. 0.5) in BALB/c exposed to DMBA and TPA as compared with TPA alone.

Urethane Initiation and TPA Promotion

Skin tumors were found primarily in SENCAR mice given urethane and TPA (Fig. 6). There were 12 mice with squamous cell carcinomas histologically, 7 of which were observed clinically. In 5 of the 12 mice with squamous cell carcinoma, 4 had metastases to lymph nodes and 2 to lungs. Vascular tumors were significantly more common in BALB/c mice (16/60) than in SENCAR mice (3/59). Most were hemangiosarcomas of the uterus and peritoneal surfaces. Uterine polypoid adenomas occurred only in BALB/c mice (6/60). The development of lung tumors were compared grossly and microscopically (Table 4). The microscopically visible lung tumors may have actually been greater since only gross lesions were sectioned. The many gross nodules in saline-treated SENCAR mice were seen histologically as probable chronic Sendai virus lesions. Histologically, tumors were alveolar, solid, tubular, or papillary adenomas and adenocarcinomas (Table 5). No difference in distribution of tumor types was affected by treatment although more papillary tumors were seen in BALB/c mice.

Selected representative morphologic lung tumors were evaluated for pulmonary antigens. Immunocytochemically, SAP was found in the cytoplasm of many tumor cells of alveolar and solid lung tumors (Figs. 7 and 8) but in few cells of tubular and papillary tumors. Rarely, tumor cell nuclei of papillary tumors contained the antigen. Clara cell antigen was never found in any lung tumors, not even in papillary tumors (Fig. 9).

TPA alone after either of the 2 vehicles caused skin papillomas and carcinomas in SENCAR mice by 52 weeks (Tables 2 and 3).

Discussion

SENCAR mice were much more susceptible to 2-stage induction of skin tumors than were BALB/c mice as previously reported (3), but developed fewer carcinogen-initiated vascular and uterine tumors than did BALB/c mice. The increased susceptibility of BALB/c mice to vascular tumors has been previously reported (7-9). The decreased susceptibility to these induced tumors in SENCAR mice cannot be readily explained. It was shown that metabolism of some carcinogens does not differ significantly in these mice (1) but their increased skin tumors susceptibility, however, may be related to the dark cell population (2). Strains and stocks of mice generally differ in their susceptibility to carcinogens because of differences in genetics, pharmacokinetics, background tumor incidences, and other factors. The glomerulonephritis and associated immunological lesions in our SENCAR mice may alter their responses to initiated cells, preneoplastic lesions or tumors (10). Skin tumors induced by ultraviolet light (UV) were found to be associated with immunological dysfunction which may be related to skin carcinogenesis (10). Since SENCAR mice are also more susceptible to UV skin carcinogenesis than other mouse strains, similar mechanisms may act in SENCAR mice. Internal tumor development in these mice may not, however, depend so much on immunological changes (12,13).

The slight, but insignificant increase in lung carcinogenesis in SENCAR mice initiated by urethane and promoted by TPA was not found in BALB/c mice. It has been reported that phorbol promoted lung tumors initiated by dimethylnitrosamine (14). We may have expected a greater tumor incidence and multiplicity in urethane/TPA mice but they died earlier than the SENCAR mice treated only with urethane. Also, Sendai virus infection, suggested in these mice by pulmonary lesions, has been shown to reduce induced lung tumor incidence (15), and may have affected the outcome of our experiment. Selected urethane-induced lung tumors usually were immunoreactive for SAP, a marker for alveolar Type II cells (6). Clara cell antigen was never detected in any lung tumors, even in papillary tumors, suggested to be of Clara cell origin (16).

The increased numbers of skin carcinomas seen histologically as compared with clinical findings is in agreement with our other studies. In two other studies with topical DMBA and TPA, we found histologically 24 carcinomas in 16 mice while only 6 were found clinically in 6 mice. The ratio of 3-4 times as many histologically appears to be consistent in our three studies. It was previously described (17) that carcinomas arise within papillomas. Thus, some of the early carcinomas arising within papillomas are obviously seen clinically as papillomas. Other malignant lesions, however, can be mistaken for papillomas. It would be prudent to section all skin tumors in studies when carcinoma diagnoses is a critical endpoint.

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Table 1. Experimental Design: The Carcinogenicity of DMBA, Urethane and TPA in Mice.

Group	Strain or Stock	# of mice initiated	Initiator[a]	Promoter[b]
1	SENCAR	15	0.25 ml DMSO/SSV	Acetone
2		15	0.25 ml DMSO/SSV	TPA
3		15	0.25 ml Saline	Acetone
4		15	0.25 ml Saline	TPA
5		30	DMBA (300 μg in 0.25 ml DMSO/SSV)	Acetone
6		30		TPA
7		30	Urethane (20 mg in 0.25 ml saline)	Acetone
8		30		TPA
9	BALB/c	15	0.25 ml DMSO/SSV	Acetone
10		15	0.25 ml DMSO/SSV	TPA
11		15	0.25 ml Saline	Acetone
12		15	0.25 ml Saline	TPA
13		30	DMBA (300 μg in 0.25 ml DMSO/SSV	Acetone
14		30		TPA
15		30	Urethane (20 mg in 0.25 ml saline)	Acetone
16		30		TPA

[a] Once intraperitoneally at 7 weeks of age.

[b] Once per week, 0.2 μl topically, 2.5 μg of TPA from weeks 1-6 and 1.25 μg of TPA from weeks 7-52.

Table 2. Tumor Incidence in Groups of 15 or 30 Female SENCAR or BALB/c Mice 52 Weeks After Intraperitoneal DMBA Initiation and with Topical TPA Promotion

	SENCAR				BALB/c			
	DSMO/SSV/ Acetone[a]	DMSO/SSV/ TPA[a]	DMBA/ Acetone[b]	DMBA/TPA[b]	DMSO/SSV/ Acetone[a]	DMSO/SSV/ TPA[a]	DMBA/ Acetone[b]	DMBA/ TPA[b]
Tumor type	Number of mice affected (%)							
Skin	0	7 (46.6)	0	23 (76)	0	0	0	0
Papilloma	0	7 (46.6)	0	18 (60)[ac]	0	0	0	0
Carcinoma	0	1 (6.6)	0	17 (56)[ac]	0	0	0	0
Sarcoma	0	0	0	2 (6)	0	0	1 (3)	1 (3)
Lung	0		11 (36)	7 (23)	1	0	10 (33)	14 (46)
Ovary	0	0	4 (13)	2 (6)	0	0	4 (13)	2 (6)
Blood vessels	0	0	5 (16)	2 (6)	0	0	15 (50)[c]	20 (67)[c]
Uterine polypoid adenoma	0		1 (3)	0	1	0	5 (16)	6 (20)[c]
Lymphoma	0	0	3 (10)	2 (6)	0	0	0	2 (6)

(continued)

Table 2 (continued)

Tumor type	SENCAR				BALB/c			
	DSMO/SSV/ Acetone[a]	DMSO/SSV/ TPA[a]	DMBA/ Acetone[b]	DMBA/TPA[b]	DMSO/SSV/ Acetone[a]	DMSO/SSV/ TPA[a]	DMBA/ Acetone[b]	DMBA/ TPA[b]
	Number of mice affected (%)							
Mammary gland carcinoma	0	1 (6.6)	2 (6)	2 (6)	0	0	0	0
Intestine	0	0	1 (3)	1 (3)	0	0	0	0
Histiocytic sarcoma	1 (6.6)	0	0	1 (3)	0	0	0	0
Any tumor	1 (6.6)	7 (46.6)	20 (67)	26 (86)	2/15 (13.3)	0	14 (80)	26 (86)

[a] 15 mice

[c] 30 mice

[c] $p < 0.05$ vs. same group for other mouse strain.

Table 3. Tumor Incidence in Female SENCAR and BALB/c Mice 52 Weeks After Intraperitoneal Urethane Initiation and/or Topical TPA Promotion

	SENCAR				BALB/c			
	Saline/ Acetone	Saline/TPA	Urethane/ Acetone	Urethane/ TPA	Saline/ Acetone	Saline/TPA	Urethane/ Acetone	Urethane/ TPA
	Number of mice examined							
		15	29	30		15	30	30
Tumor type	Number of mice with tumors (%)							
Skin	0	1 (6.6)	1 (3)	20 (67)^{c}	0	0	0	1 (3)
Papillomas	0	1 (6.6)	0	15 (50)^{c}	0	0	0	1
Carcinomas	0	0	1 (34.4)	12 (40)^{c}	0	0	0	0
Lung	1 (6.6)	1 (6.6)	18 (62)	24 (80)	2 (13.3)	2 (13.3)	26 (92)^{a}	29 (100)^{b}
Blood Vessels	0	0	2 (6)	1 (3)	0	0	9 (30)^{c}	7 (23)^{c}
Hemangioma	0	0	1	1	0	0	2	5
Hemangiosarcoma	0	0	1	0	0	0	7	2
Hematopoietic system	0	0	3 (10)	1 (3)	0	1 (6.6)	0	0

(continued)

Table 3 (continued)

	SENCAR				BALB/c			
	Saline/ Acetone	Saline/TPA	Urethane/ Acetone	Urethane/ TPA	Saline/ Acetone	Saline/TPA	Urethane/ Acetone	Urethane/ TPA
	Number of mice examined							
		15	29	30		15	30	30
Tumor type	Number of mice with tumors (%)							
Forestomach	0	0	2 (6)	0	0	0	0	0
Uterus-	0							
leiomyosarcoma	0	0	1 (3)	0	0	0	0	0
polypoid adenoma	0	1 (6.6)	0	0	0	3 (20)	3 (10)	3 (10)
histiocytic sarcoma	0	2 (13.3)	0	0	0	0	0	0
Any tumor	1 (6.6)	5 (33.3)	23 (79.3)	24 (80)	2 (13.3)	5 (35.7)	26 (86.6)	29 (96.6)

[a] From 28 lungs available for histology.

[b] From 29 lungs available for histology.

[c] $p < 0.05$ vs. same group for other mouse strain.

Table 4. A Comparison of Gross and Microscopic Pulmonary Lesions in Female SENCAR and BALB/c Mice 52 Weeks After Urethane Initiation and Topical TPA Promotion.

	SENCAR			BALB/c		
	Saline/ Acetone	Urethane/ Acetone	Urethane/ TPA	Saline/ Acetone	Urethane/ Acetone	Urethane/ TPA
	Number of mice examined					
	15	29	30	15	30	30
Number of mice with:	Number of mice with tumors (%)					
Histologic lung tumors	1 (7)	18 (62)	24 (80)	2 (13)	26 (92)	29 (100)
Gross lung tumors	7 (46.6)	17 (58.6)	24 (80)	1 (6.6)	26 (92)	26 (92)
Gross lung nodules per mouse initiated[a]	1.3 ± 0.57	3.3 ± 0.79	4.3 ± 0.79	0.07 ± 0.07	4.5 ± 0.77	3.4 ± 0.43
Histologic tumors per mouse initiated[a]	0.07 ± 0.07	2.1 ± 0.52	2.7 ± 0.5	0.13 ± 0.09	4.6 ± 0.71	4.4 ± 0.39

[a] Mean ± S.E.M.

Table 5. Histologic Types of Lung Tumors in Female SENCAR and BALB/c Mice Initiated with Urethane and Topical TPA Promotion.

	Number of tumors (%)	
Tumor type	SENCAR	BALB/c[a]
All tumors	145 (100)	259 (100)
Alveolar, solid or mixed	68 (46.9)	79 (30.5)
Solid/tubular or tubular	32 (22.1)	71 (27.4)
Tubular/papillary or papillary	45 (31.0)	109 (42.1)

[a] Urethane/acetone and urethane/TPA groups combined.

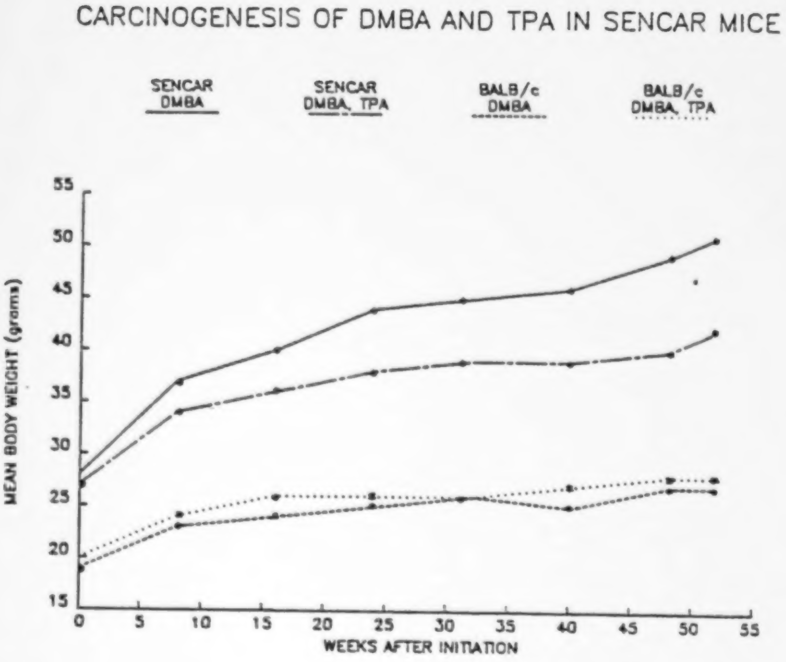

FIGURE 1. Growth of female SENCAR mice exposed to DMBA and TPA.

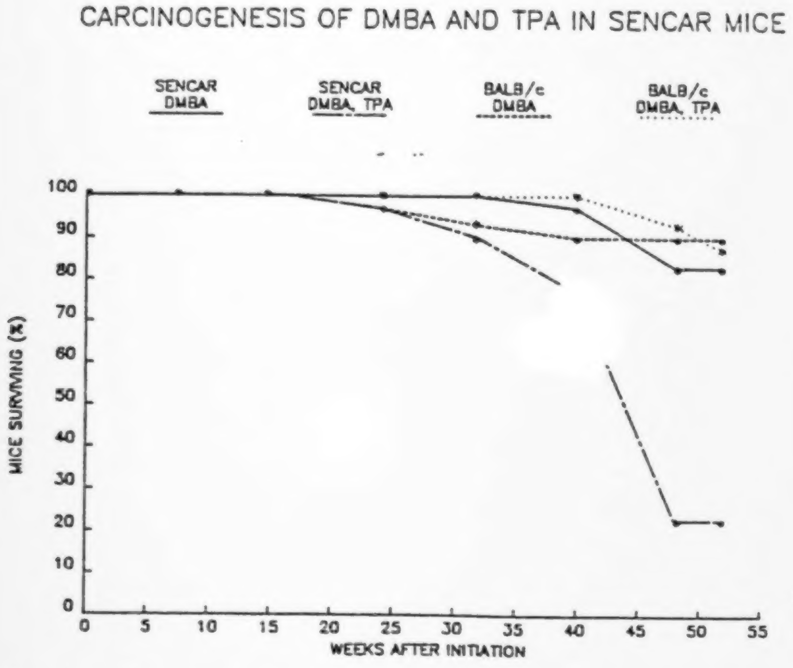

FIGURE 2. Survival of female SENCAR mice exposed to DMBA and TPA.

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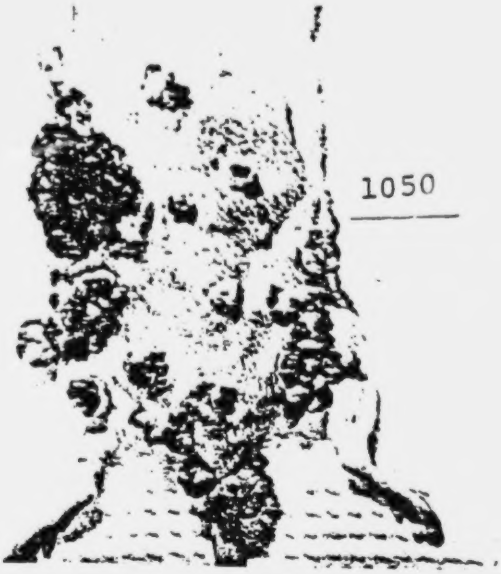

FIGURE 3. Multiple skin papillomas and one carcinoma in a SENCAR mouse initiated with DMBA and promoted by TPA.

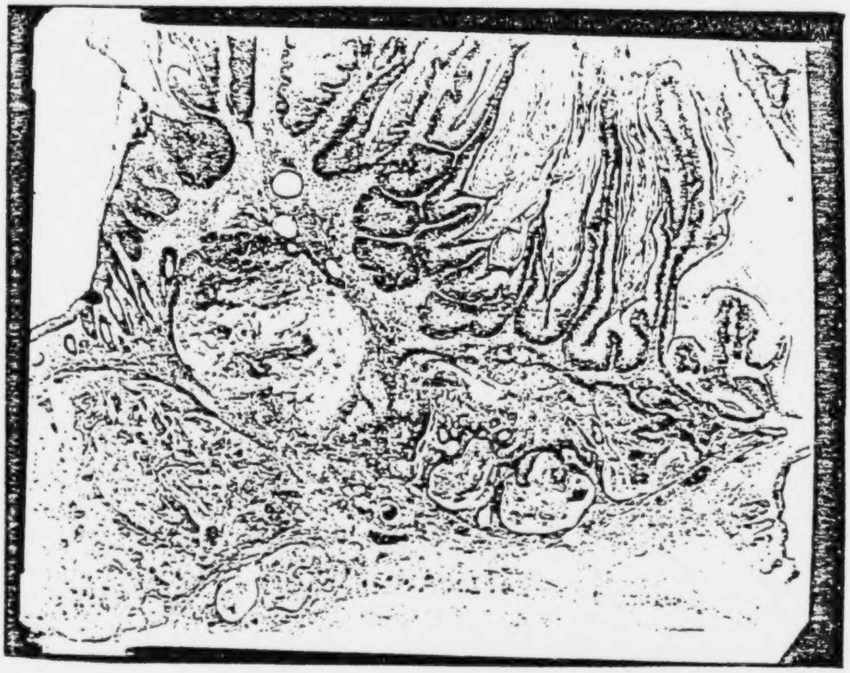

FIGURE 4. Squamous cell carcinoma arising within base of papilloma after exposure to DMBA and TPA (H & E, x 25).

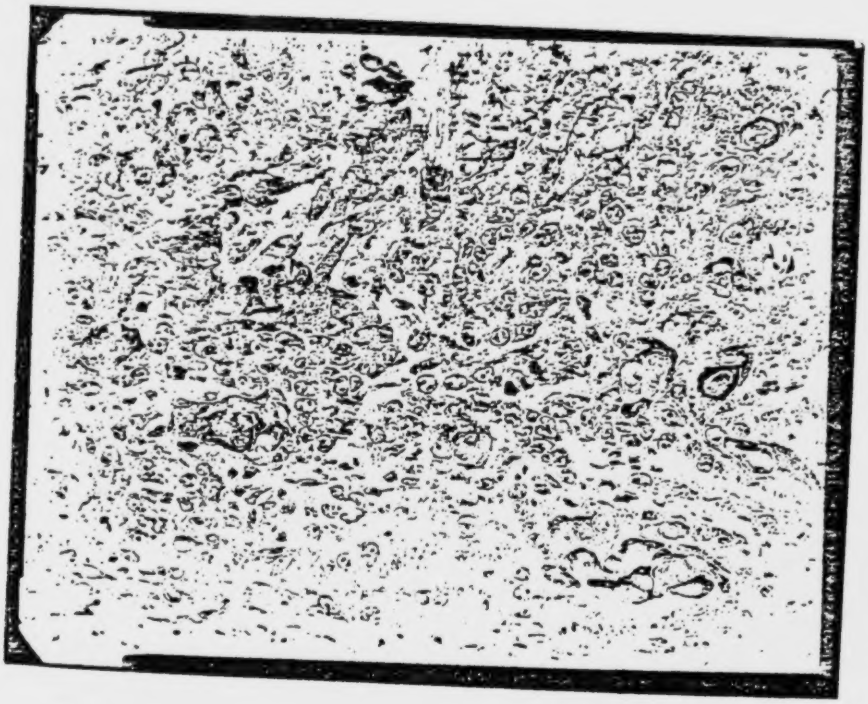

FIGURE 5. Keratin in poorly differentiated squamous cell carcinoma after trypsin digestion of tissue section (ABC immunoperoxidase, hematoxylin, x 250).

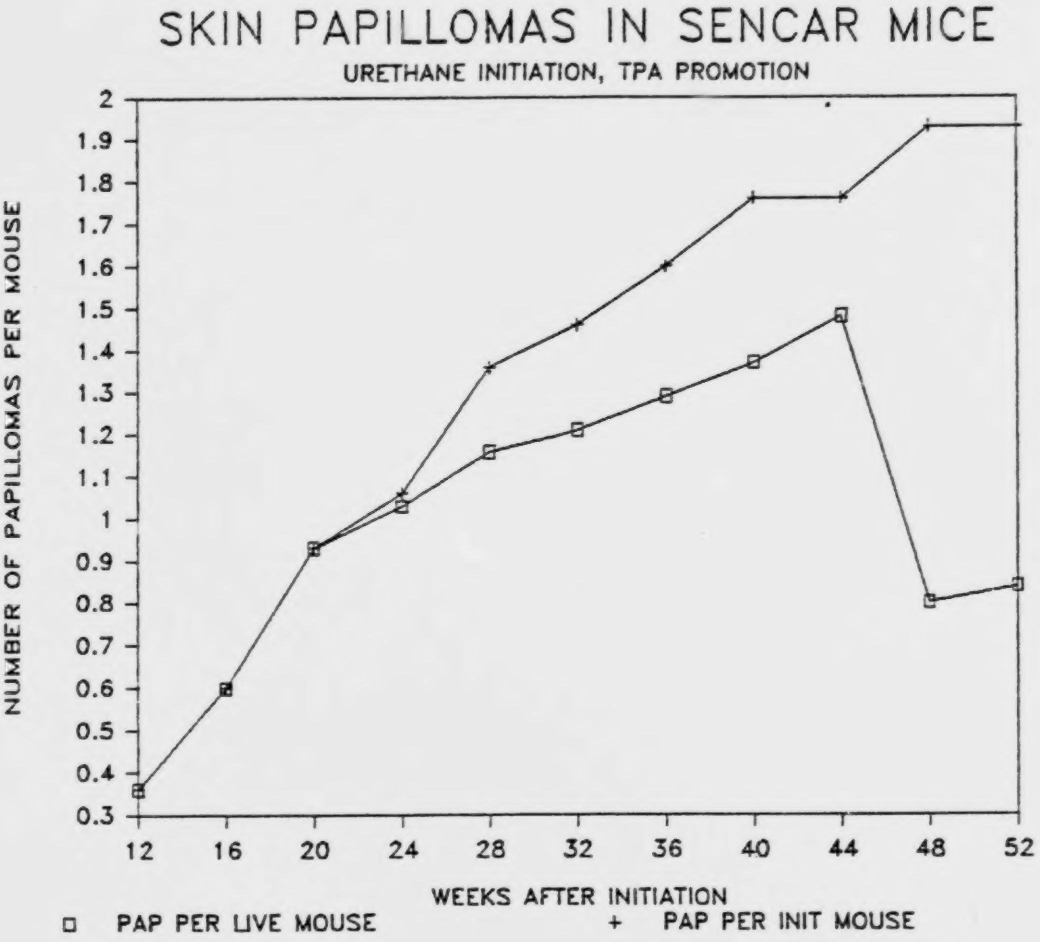

FIGURE 6. Skin papillomas in female SENCAR mice initiated by 20 μg of urethane intraperitoneally at 7 weeks of age and promoted topically for 1-6 weeks later with TPA at 2.5 μg per mouse and then 1.25 μg per mouse from weeks 7-52.

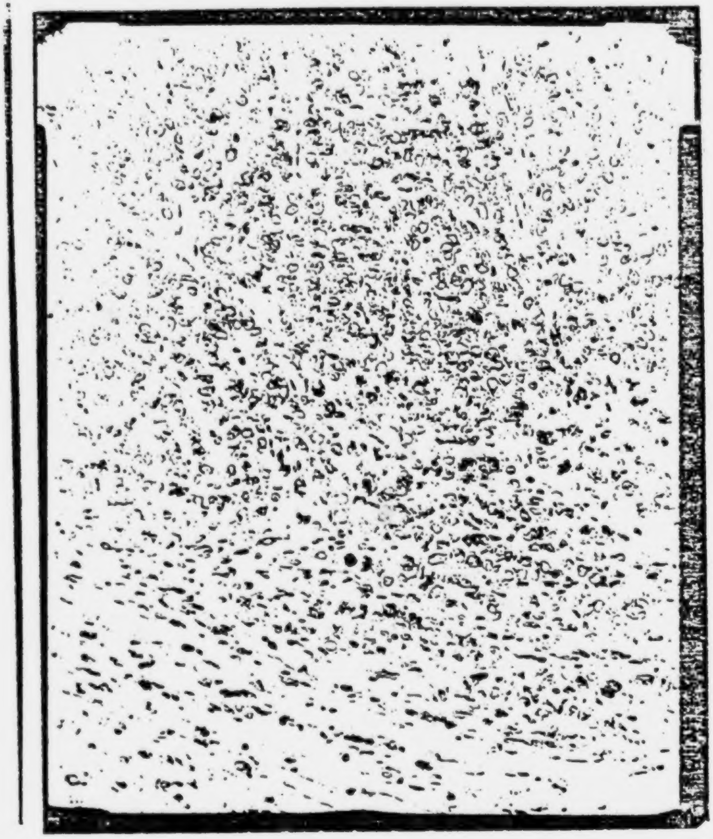

FIGURE 7. Surfactant apoprotein in normal alveolar Type II cells (bottom of figure) and few tumor cells in solid-tubular pulmonary adenomas induced by urethane (ABC immunoperoxidase, hematoxylin, x 250).

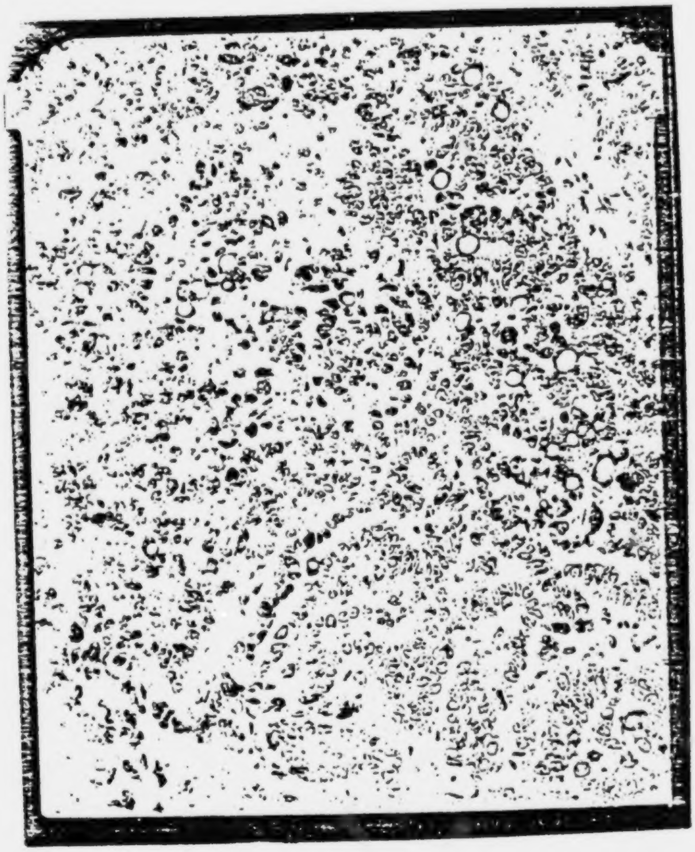

FIGURE 8. Surfactant apoprotein in vacuolated tumor cells in solid areas of pulmonary adenoma and not in tubulary focus (ABC immunoperoxidase, x 250).

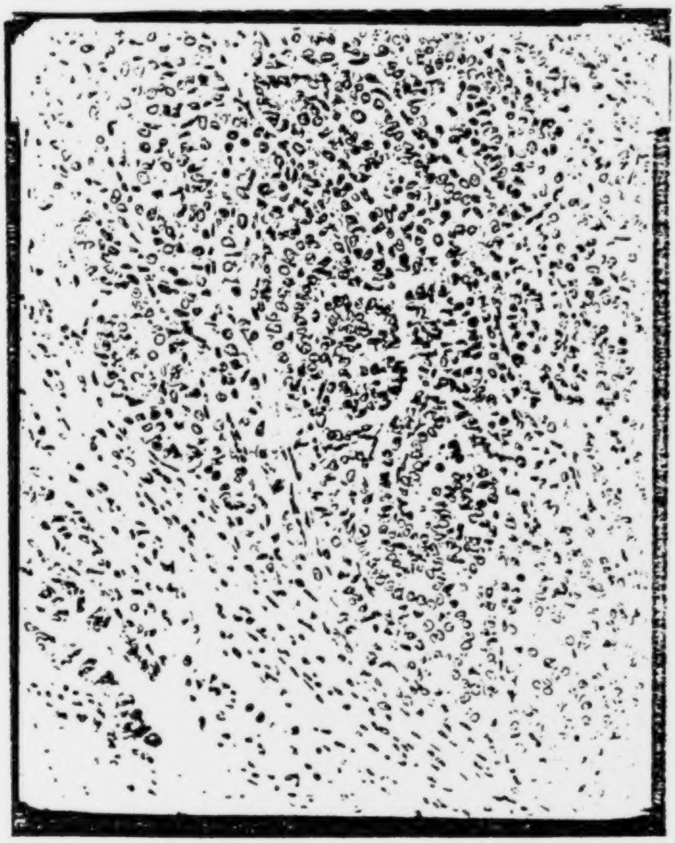

FIGURE 9. Clara cell antigen in normal Clara cells in bronchiole (arrow) but not in tubulopapillary pulmonary adenoma (ABC immunoperoxidase, hematoxylin, x 250).

PATHOLOGY OF AGING FEMALE SENCAR MICE USED AS CONTROLS IN SKIN 2-STAGE CARCINOGENESIS STUDIES

by

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Key Words: SENCAR mouse, Glomerulonephritis, Immune complex disease, Histiocytic sarcoma, TPA, Skin tumors, Avidin-biotin immunocytochemistry.

Abstract

The pathology of 60 aged female SENCAR mice used as acetone controls in skin painting studies was studied. Fifty percent of the mice survived past 96 weeks of age. The major known contributing causes of death of 42 mice were glomerulonephritis (8 mice), histiocytic sarcoma (7), and other tumors (8). Glomerulonephritis was found in the majority of mice and was associated with thymic hyperplasia, focal vasculitis and lymphoid hyperplasia. Necropsy of 58 mice surviving past 50 weeks of age revealed 41 had an average of 1.36 tumors per mouse. The most common tumors included histiocytic sarcoma (13 mice), pulmonary adenoma or adenocarcinoma (11), mammary tumors (11), follicular center cell lymphoma (4), and hepatocellular adenoma (4). The 13 histiocytic sarcomas appeared to arise in the uterus and metastasized to liver (9 mice), lung (4), kidney (3), and other tissues. Lung tumors were of the solid and papillary types and tumor cells frequently contained surfactant apoprotein but not Clara cell antigens suggesting their alveolar Type II cell origin. A variety of nonneoplastic lesions were seen in other tissues of these mice which were similar to those observed in other mouse strains. Amyloidosis was seen only in nasal turbinates and thyroid gland.

In a group of 28 mice exposed to 12-0-tetradecanolyphorbol-13-acetate (TPA) for up to 88 weeks, as a control for other treatment groups, 7 (25%) had papillomas and 5 (17.8%) had squamous cell carcinomas of the skin at necropsy, although many other induced papillomas regressed during the study.

Introduction

SENCAR mice were bred to increase sensitivity to skin initiation and promotion protocols (1-3). They originated in 1959 from Rockland all-purpose mice which were inbred for sensitivity to skin initiation by 7,12-di-methylbenz[a]anthracene (DMBA) and promotion by croton oil. In 1971, these susceptible mice were outbred with Charles River CD-1 mice to produce hybrid vigor. These mice have been bred at Oak Ridge National Laboratory for use in skin carcinogenesis studies of 6 to 12 month duration. There have been no reports of the pathology of aging SENCAR mice, although several studies of the CD-1 mouse have been published (4,5). As part of a large study to characterize the possible skin initiating and promoting activities of formaldehyde (6), we maintained groups of female SENCAR mice for up to 100 weeks of age. There was no evidence that any of the tumors or lesions in these mice were associated with their treatment with acetone, and were considered to occur naturally. In this paper, we describe the pathology of these aged mice.

Materials and Methods

For a large study to test the skin initiating or promoting properties of formaldehyde, 260 female SENCAR mice were obtained from Oak Ridge National Laboratory Animal Resources (Oak Ridge, TN) at 7 weeks of age. Sixty of these were used for the present study. They were placed in polycarbonate cages, 11.5 x 7.5 x 5 inches in size, on corn cob bedding. Water and Purina Lab chow were available ad lib. For 30 mice of treatment group 4B, 0.2 ml of a 10% formalin solution (37 to 40% formaldehyde solution in acetone) was applied once to the back at 8 weeks of age. Four weeks later 0.2 ml acetone was applied once weekly for 88 weeks. For 30 mice of experimental group 4D, 0.2 ml of acetone was applied twice weekly to their backs from 8 weeks of age for 92 weeks. These mice were weighed monthly. No untreated mice were available for aging studies. These groups are combined because all nonneoplastic and nonneoplastic lesions occurred in similar incidences in both groups and survival was similar. All surviving mice were sacrificed at 101-102 weeks of age, 93-94 weeks after initiation of the study.

After arrival from the supplier and at terminal sacrifice (100 weeks of age), serum samples were obtained from 2 and 15 mice, respectively, sent to the Comprehensive Health Service of Microbiological Associates (Bethesda, MD), and tested by standard procedures by hemaglutination-inhibition (HI) for antibodies to the following viruses: PVM; Sendai; Reo 3; K; polyoma; MVM; and GDVII, by complement fixation for antibodies to mouse adenovirus and LCM and by ELISA for antibodies to MVM, PVM, Sendai, Ectromelia, and GDVII.

A complete necropsy examination was performed on each mouse. The majority of the major organs and gross lesions were fixed in 10% neutral buffered formalin. All major tissues, gross lesions and selected other tissues were embedded in paraffin and sections were prepared to 4 to 6 M and stained with hematoxylin and eosin. For selected tissues, the avidin biotin peroxidase-complex (ABC) immunocytochemical technique was used (7,8) with the Vectastain kits (Vector Laboratories, Burlingame, CA) to localize specific antigens with the use of antibodies to surfactant apoprotein (SAP) (7), Clara cell antigen (7), prolactin (National Hormone and Pituitary Program, Baltimore, MD), insulin (Miles Laboratories, Elkhart, IN), glucagon (DAKO Corp., Santa Barbara, CA), S-100 (DAKO Corp.), somatostatin (DAKO Corp.), lysozyme (DAKO Corp.), alpha-1-antitrypsin (DAKO Corp.), biotinylated anti-mouse IgG (Vector Laboratories), mouse lambda and kappa immunoglobulin light chains (Litton Bionetics, Charleston, SC), or vimentin (Transformation Research, Inc., Framingham, MA). For localization of immunoglobulins or immunoglobulin light chains, tissue sections were trypsinized first.

Portions of selected formalin-fixed kidneys were placed in glutaraldehyde, embedded in epoxy resin, sectioned and stained with uranyl acetate and lead citrate and viewed under an electron microscope.

Results

Body Weights, Serology and Survival

The mean body weight curve of mice in groups 4B and 4D is shown in Figure 1. The mean weight of mice steadily rose throughout their lifespan reaching 49 grams at 100 weeks of age. While two mice died or were sacrificed prior to 16 weeks of age, the next one died at 52 weeks of age and 50% survived to 96 weeks of age (Fig. 2). Forty-four mice died during the study.

Serology performed on the 2 mice at the time of arrival from the supplier did not contain murine viral antibodies. At the end of the study, sera from 15 aged mice contained significant serum viral antibodies titers by ELISA to MHV, (14/15), Sendai virus (6/6), MVM (6/6); by HI to PVM (2/15), Sendai virus (12/15), and MVM (10/15). Other serological tests were negative.

Pathology

Contributing Causes of Death

The major contributing causes of illness or death of 42 mice are noted in Table 1. These determinations were based on gross and microscopic findings. Thirty-five percent of these mice died from the effects of tumors and 64 percent died from other causes. Glomerulonephritis was a major cause of death for 8 mice, while 2 of these mice had generalized edema (nephrotic syndrome). Histiocytic sarcoma arising in the uterus and spreading to other tissues was the major lethal tumor type.

Neoplastic and Nonneoplastic Lesions

Forty-one (70.6%) of the 58 mice surviving past 52 weeks of age had an average of 1.36 tumors per mouse (Table 2). Many mice had a variety of nonneoplastic lesions as shown in Table 3. The major lesions and some unusual lesions are described according to organ system. One mouse receiving acetone twice weekly for 88 weeks had a papilloma at the site of application which appeared only at 92 weeks.

Kidney Lesions. The primary renal lesion consisted of glomerulonephritis with a variety of secondary lesions. Glomerular lesions were scored mild, moderate or severe depending on their extent and severity. In general, within a given kidney, glomeruli exhibited a variety of changes consisting of increased mesangial matrix and mesangial cells, thickened capillary loops, sclerosis, and hypertrophy of mesangial cell nuclei (Fig. 3). Some glomeruli were involved in more acute processes characterized by fibrin deposition, mild neutrophilic infiltrate, and individual cell necrosis. A few cases had crescent formation, fibrosis, and obliteration of glomerular tufts. Immunocytochemically, we demonstrated mouse IgG and immunoglobulin kappa light chains within thickened glomerular capillary loops and within the mesangium (Fig. 4) after trypsinization of formalin fixed kidney sections. Ultrastructurally, subendothelial and subepithelial electron dense deposits were seen within thickened glomerular basement membranes (Fig. 5). There was also obliteration of epithelial foot processes.

Severe tubular degeneration and hyaline cast formation, although present in several animals, was not a prominent feature in most mice. The majority of mice, however, did have mild to marked lymphocytic and plasma cell perivascular cuffs around intrarenal arteries.

Hematopoietic System

Follicular center cell (B cell) lymphomas of spleen and lymph nodes were found in 4 mice. With trypsinization of formalin-fixed tissue sections, immunoglobulin was seen focally within tumor cells. The primary nonneoplastic lesions involving the lymphoreticular system consisted of mild to severe lymphoid cell hyperplasia of both B- and T-cell zones. The thymuses in nearly all mice were very prominent (excessively so for 1 to 2 yr old mice) and had a mixture of cortical and medullary (follicular) lymphoid cell hyperplasia together with the proliferation of medullary epithelial cells and sometimes with small cyst formation. The spleens exhibited varying degrees of periarterial lymphoid sheath hyperplasia with associated lymphoid follicles. Also, the amount of myeloid metaplasia in the white pulp was frequently excessive. Lymph nodes similarly exhibited follicular and paracortical hyperplasia together with varying degrees of plasmacytosis. Myeloid metaplasia was also present in several nodes as well. These mice did not have induced skin tumors.

Liver

Four hepatocellular adenomas and two basophilic foci were found in these mice. Generally, all nonneoplastic lesions noted were very mild. The patterns of focal necrosis varied from individual cell to larger discrete portions of hepatic lobules, suggestive of ischemia. Livers with individual cell necrosis often had concurrent mild focal dense infiltrates of neutrophils or macrophages forming small discrete granulomas. Livers with ischemic necrosis often had marked tumor infiltrate. All inflammatory lesions were similarly mild. The chronic inflammatory lesions consisted primarily of small dense focal infiltrates of lymphocytes with lesser numbers of plasma cells in portal areas around central veins or in parenchyma.

Respiratory Tract

Lung tumors were solid and papillary (Table 2). The tumor cells frequently contained surfactant apoprotein (SAP), but never Clara cell antigen when stained by the ABC technique. Of 2 solid tumors, 2 were immunoreactive for SAP; of 5 papillary tumors, 1 had foci of intranuclear reactivity for SAP and of 3 tubular or solid-tubular tumors, 2 were immunoreactive for SAP. Nonneoplastic pulmonary lesions were generally mild. Peribronchial and perivascular lymphoid hyperplasia were nonspecific and typical of lesions present in many old rodents. Interstitial pneumonia was often associated with lymphoid cuffs around bronchioles and blood vessels as well as a small multifocal areas of thickened alveolar septae with mononuclear inflammatory cells and prominent alveolar Type-II cells. Some of these foci may have represented resolving Sendai virus infections. One animal with interstitial pneumonia also had active bronchiolitis. A high incidence (78%) of apparent amyloidosis of the nasal turbinates was seen (Fig. 6). This hyaline material, however, did not stain with crystal violet or Congo red.

Reproductive Tract

Histiocytic sarcoma (Reticulum cell sarcoma, Type A; lymphoma, histiocytic) was the major cause of death (7 mice) and apparently metastasized to several organs (Fig. 7). Thirteen mice (22.4%) had this tumor. A few early histiocytic sarcomas were found in the uterus. We found alpha-1-antitrypsin in some tumor cells (Fig. 8) of a few tumors, by ABC immunocytochemistry, but never S-100, lysozyme or vimentin. One tumor was easily transplanted and cultivated *in vitro*. The rounded tumor cells grew very slowly *in vitro* but grew quickly into tumors when transplanted *in vivo*.

Uterine cystic endometrial hyperplasia was typical of that found in old rodent females and varied from mild to severe with complete distortion of uterine architecture. One mouse had a severe necrotizing metritis which contributed to its early mortality. Ovarian cysts were present in nearly all animals and ranged from small cysts to very large thin-walled structures with complete loss of normal ovarian tissue. In many animals, a large cystic mass was distended with clotted blood often reaching up to 1 cm in diameter. On the edge of a few of these hemorrhagic lesions was focal angiectasis.

Thirteen mammary tumors were found in 11 mice. After trypsinization and ABC immunocytochemistry, keratin was found in some tumor cells of several tumors, especially in focal areas of squamous metaplasia in the adenocanthoma (Fig. 9).

Cardiovascular System

The vasculitis present involved, primarily, medium size to large arteries, especially the ascending aorta. The distribution of inflammatory cells varied from multifocal to focally extensive infiltrates extending from the adventitia into the muscularis with a pattern typical of periarteritis nodosa. The perivascular inflammatory infiltrate consisted of small lymphocytes, lymphoblastoid cells, macrophages, a few plasma cells, and neutrophils. The adjacent media exhibited varying degrees of hypertrophy and hydropic degeneration of smooth muscle cells and fibrinoid necrosis. In several animals, this inflammatory necrotizing process resulted in fatal rupture of the vessel wall.

Endocrine System

Few mice had endocrine tumors (Table 2). The 3 pituitary adenomas contained abundant immunoreactive prolactin (Fig. 10). One tumor adhered to and/or invaded the brain parenchyma (Fig. 10). Pancreatic islet cell hyperplasia was seen in 35.4% of the mice. The enlarged islets contained diffuse immunoreactive insulin and focal somatostatin and glucagon-containing cells (Fig. 11), in a pattern identical to that of normal islets.

The Carcinogenesis of 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) in Female SENCAR mice

As part of our large skin initiation-promotion studies of formaldehyde, we had included a group of 30 mice which received TPA for 88 weeks. At 8 weeks of age, these 30 mice received 0.2 ml acetone topically on the back. From 12 weeks of age, they received once weekly 1.25 µg of TPA (Dr. P. Borchert, Eden Prairie, MN) for up to 88 weeks. Mice were sacrificed at 100 weeks of age and a complete necropsy was performed. Acetone controls were reported above, as our aged acetone control mice. The clinical findings for skin papillomas and carcinomas are shown in Fig. 12. Many of the clinical papillomas apparently regressed. The mean body weights of the mice in this group was similar to those receiving only acetone. Two mice died or were sacrificed prior to week 24. Sixty percent of the mice (18/30) survived past week 88 after acetone initiation (96 weeks of age). Thirteen of the 30 mice were still alive at terminal sacrifice (100 weeks of age). Histologically at the time of necropsy, there were 7 mice with papillomas (of 28 mice surviving past 48 weeks - 25%) and 5 (17.8%) with squamous cell carcinomas (including 2 poorly differentiated carcinomas with areas of sarcoma) at necropsy. At least two of these carcinomas were seen to arise clinically from papillomas and histological evidence of this was observed (Fig. 13). Several skin lesions were recorded as possible papillomas or plaques were seen which may have been regressing lesions noted clinically (Fig. 14). These mice also had a variety of other tumors including those of the lung (4 mice), pituitary (2), hemangiosarcoma (3), Harderian gland (2), follicular center cell lymphoma (3), histiocytic sarcoma (2) mammary gland (1), and miscellaneous tissues (5). Two of the mice had liver, splenic, and intestinal amyloidosis.

Discussion

Female SENCAR mice used as controls in skin painting studies were quite hardy with more than 50% surviving to 96 weeks of age, despite having serological evidence of exposure to Sendai virus and mouse hepatitis virus. Pulmonary lesions were also compatible with resolving or chronic Sendai virus lesions. The longevity of our mice appeared to be considerably longer than for CD-1 mice, a parenteral strain for SENCAR mice (5,9). The increased survival may have been due to a low incidence of generalized amyloidosis, a major lesion in CD-1 mice (4,9). The tumors that were found in our SENCAR mice occurred in similar incidence to CD-1 mice with some differences (5,9). The histiocytic sarcomas, mammary tumors, and lung tumors were common in both stocks of mice.

The glomerulonephritis was unexpected in SENCAR considering the lack of this important disease in CD-1 mice. We did, however, find amyloid in the nasal cavity and thyroid. Glomerular lesions were not as severe as seen in some lupus-like syndromes but resembled lupus because of electron dense deposits in glomerular basement membranes, vasculitis, thymic hyperplasis (10,11) and nephrotic syndrome in a few mice. The association between CD-1 mouse amyloidosis, abnormal immunoglobulin synthesis and glomerulonephritis in SENCAR mice may be related to defective immune functions in these stocks of mice. The possible important role of the immune system in ultraviolet skin carcinogenesis in mice (12,13) suggests that the immune dysfunction in SENCAR mice may also be important for chemical carcinogenesis of the skin (14).

Female SENCAR mice appear to have a very high incidence of histiocytic sarcoma. This unusual tumor has been described as reticulum cell sarcoma Type A (15), histocytic lymphoma (16,17), and malignant schwannoma (18).

In female mice, tumors often arise within the uterus. The specific cell of origin has not been described but tumor cells have been described to have histochemical and ultrastructural evidence of histiocytic differentation (16). We could not localize common histiocytic antigens including lysozyme and vimentin and the Schwann cell marker antigen S-100. We found alpha-1-antitrypsin only ocasionally within tumor cells. Additional studies should clarify the origin of this tumor.

TPA was previously reported to cause skin papillomas (1,2,19-23) and carcinomas (19) in SENCAR mice. TPA was first reported to cause skin papillomas in other strains of mice (24-27). Carcinomas were reported in fewer studies (24).

Islet cell hyperplasia has been associated with obesity (28). The finding of insulin, glucagon, and somatostatin in anatomical patterns similar to those in normal islets differentiates the enlarge islets from adenomas which would have single hormones or multiple hormones in an abnormal anatomical distribution (29).

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Table 1. Contributing causes of death of 42 aging female SENCAR mice

Lesion	No. affected (%)
Neoplastic	15 (35.7)
Histiocytic sarcoma	7 (16.6)
Lymphoma	3 (7.1)
Pulmonary adenocarcinoma	2 (4.7)
Mammary adenocarcinoma	2 (4.7)
Pituitary adenoma	1 (2.3)
Nonneoplastic	27 (64.3)
Glomerulonephritis	8 (19.0)
Hemoperitoneum	3 (7.1)
Necrotizing enteritis	2 (4.7)
Peritonitis	1 (2.3)
Necrotizing metritis	1 (2.3)
Hemothorax	1 (2.3)
Cachexia	1 (2.3)
Septicemia	1 (2.3)
Undetermined	9 (21.4)

Table 2. Neoplastic lesions in 58 aging female SENCAR mice

Tissue	Tumor	No. Affected (%)
Hematopoietic		
Lymphoma (Follicular center cell)		4 (6.8)
	Spleen	4
	Lymph Nodes	4
	Thymus	3
Lymphoma (other)		1 (1.7)
Reproductive tract		
Histiocytic sarcoma		13 (22.4)
	Uterus	13
	Liver	9
	Lung	4
	Kidney	3
	Lymph node	2
	Ovary	2
	Pancreas	1
	Small intestine	1
	Adrenal	1
Uterus	Leiomyosarcoma	2 (3.4)
	Endometrial polyp	2 (3.4)
	Fibroma	1 (1.7)
	Adenocarcinoma	1 (1.7)

(continued)

Table 2. (continued)

Tissue	Tumor	No. Affected (%)
Lung	Primary tumors	11 (18.9)
	Adenoma	
	Solid	6
	Papillary	3
	Adenocarcinoma	
	Papillary	2
	Metastatic tumors	
	Mammary adenocarcinoma	2 (3.4)
Endocrine	Pituitary gland	
	Adenoma	3 (5.1)
	Thyroid gland	
	Follicular adenoma	2 (3.4)
	Adrenal gland	
	Cortical carcinoma	1 (1.7)
	Pheochromocytoma	1 (1.7)
GI tract	Tongue	
	Papilloma	1 (1.7)
	Stomach	
	Polypoid adenoma	2 (3.4)
	Small intestine	
	Adenoma	1 (1.7)

(continued)

Table 2. (continued)

Tissue	Tumor	No. Affected (%)
Liver	Hepatocellular adenoma	4 (6.8)
	Hemangiosarcoma	1 (1.7)
Skin	Hemangioma	1 (1.7)
	Papilloma	1 (1.7)
Mammary gland	Adenocarcinoma	11 (18.9)
	Type A	4
	Type B	5
	Adenoacanthoma	1
	Fibrosarcoma	2
	In situ ductal carcinoma	1
Harderian Gland		
	Adenoma	3 (5.1)

Table 3. Nonneoplastic lesions of 58 aging female SENCAR mice

Tissue	Lesion	No. affected/No. tissues examined histologically (%)
Kidney	Glomerulonephritis	48/58 (82.7)
	Mild	14/58 (24.1)
	Moderate	20/58 (34.4)
	Severe	14/58 (24.1)
	Perivascular chronic inflammation	39/58 (67.2)
Hematopoietic		
Thymus	Lymphoid & epithelial hyperplasia	34/38 (89.4)
	Medullary cyst	12/38 (31.5)
Spleen	Myeloid metaplasia	46/57 (80.7)
	Lymphoid hyperplasia	36/57 (63.1)
	Hemosiderosis	5/57 (8.7)
Lymph nodes	Lymphoid hyperplasia	23/38 (60.5)
	Plasmacytosis	9/42 (21.4)
	Myeloid metaplasia	5/42 (11.9)
	Sinus histiocytosis	2/42 (4.7)
Liver	Cytomegaly	36/56 (64.2)
	Coagulative necrosis, focal	11/56 (19.6)
	Chronic cholangitis	34/56 (60.7)
	Acute cholangitis	8/56 (14.2)

(continued)

Table 3. (continued)

Tissue	Lesion	No. affected/No. tissues examined histologically (%)
Liver (cont.)	Granulomas	9/56 (19.6)
	Mitotic figures	5/56 (8.9)
	Fatty change	2/56 (3.5)
	Biliary cyst	3/56 (5.3)
	Basophilic hepatocellular focus	2/56 (3.5)
Respiratory tract	Nasal turbinates	
	Amyloidosis	18/23 (78.2)
	Chronic inflammation	3/23 (13.0)
	Lung	
	Perivascular lymphoid hyperplasia	19/56 (33.9)
	Peribronchiolar lymphoid hyperplasia	15/56 (26.7)
	Interstitial pneumonia	7/56 (12.5)
	Bronchiolitis	1/56 (1.7)
Reproductive tract	Uterus	
	Cystic endometrial hyperplasia	44/53 (83.0)
	Necrotizing metritis	1/53 (1.8)

(continued)

Table 3. (continued)

Tissue	Lesion	No. affected/No. tissues examined histologically (%)
Reproductive tract	Ovary	
	Simple cyst	20/22 (90.9)
	Hematoma	1/22 (4.5)
Endocrine	Adrenal gland	
	Subcapsular spindle cell hyperplasia	34/44 (77.2)
	Focal cortical hyperplasia	4/44 (9.0)
	Thyroid gland	
	Amyloidosis	3/38 (7.8)
	Lacrimal gland	
	Dacryoadenitis	9/13 (69.2)
	Pancreas	
	Islet cell hyperplasia	17/48 (35.4)
	Inflammation	8/48 (16.6)
	Acinar cell vacuolation	2/48 (4.1)
	Atrophy	1/48 (2.0)
	Inflammation, islets	1/48 (2.0)

(continued)

Table 3. (continued)

Tissue	Lesion	No. affected/No. tissues examined histologically (%)
Blood vessels	Vasculitis	18/56 (32.1)
	Arteritis, aorta	10/56 (17.8)
	Aorta, rupture	1/56 (1.7)
Heart		
	Fibrosis, focal	5/56 (8.9)

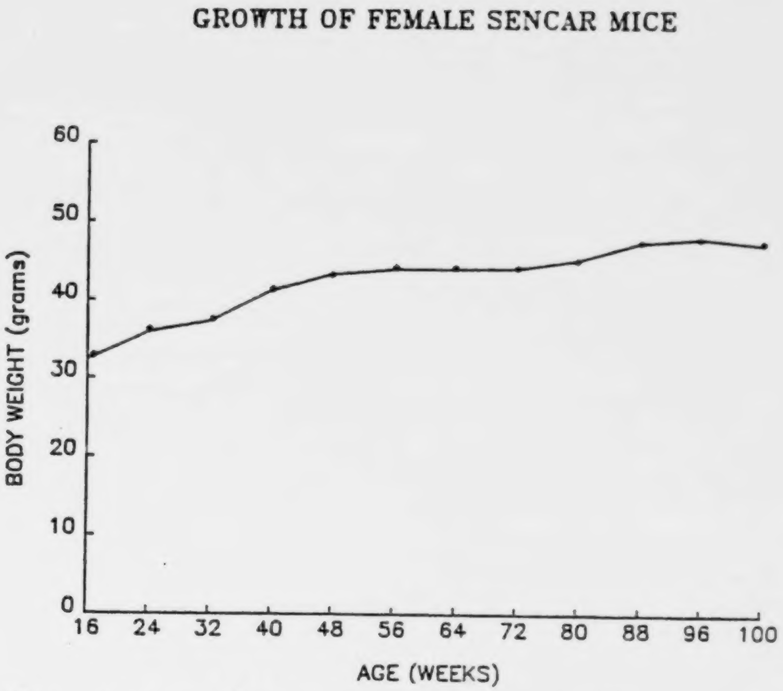

FIGURE 1. Mean body weight gain of 60 female SENCAR mice used as acetone controls in a skin initiation promotion experiment.

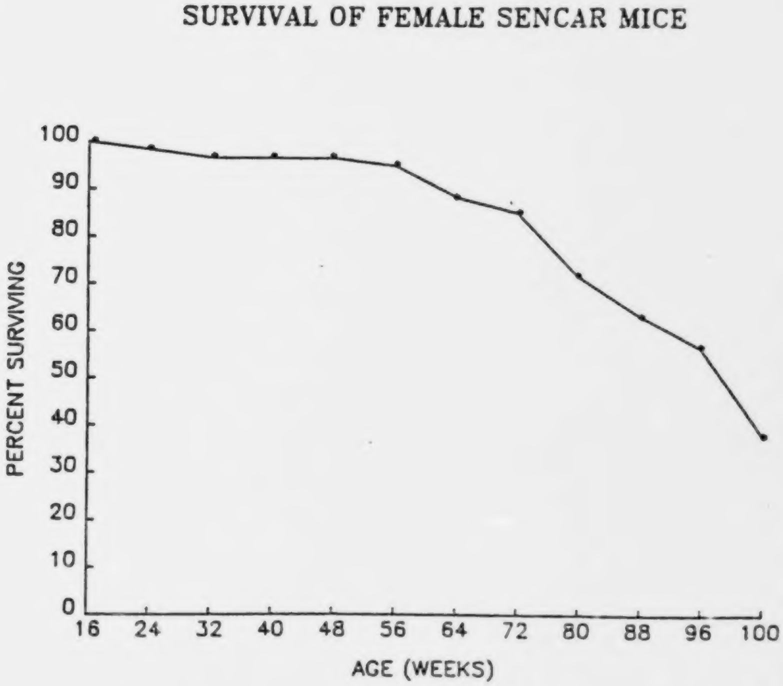

FIGURE 2. Survival of 58 female SENCAR mice living past 16 weeks of age. Fifty-percent survived to 96 weeks of age.

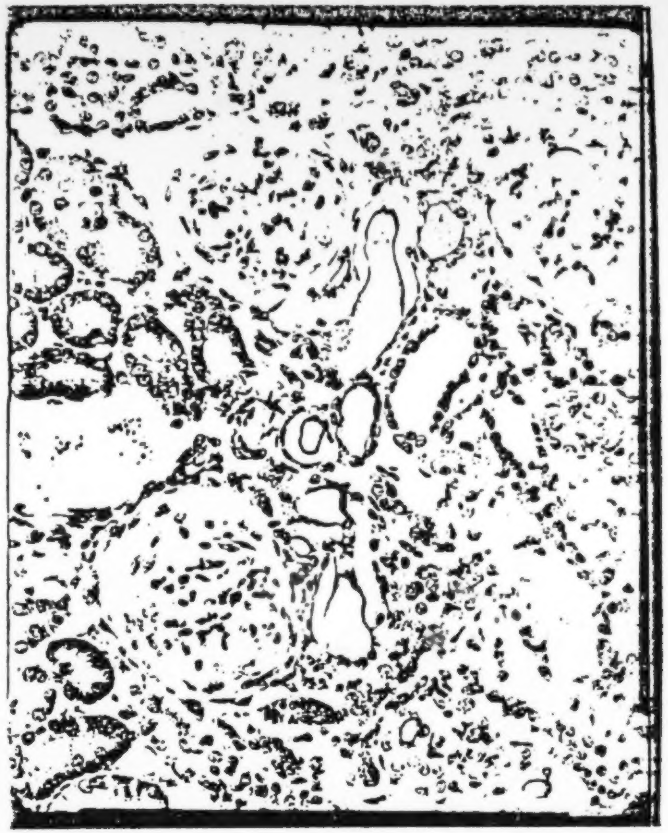

FIGURE 3. Glomerulonephritis in a female SENCAR mouse, H & E, x 250.

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FIGURE 4. Kappa immunoglobulin light chains concentrated in glomerular capillary loops and mesangium. H & E, x 400.

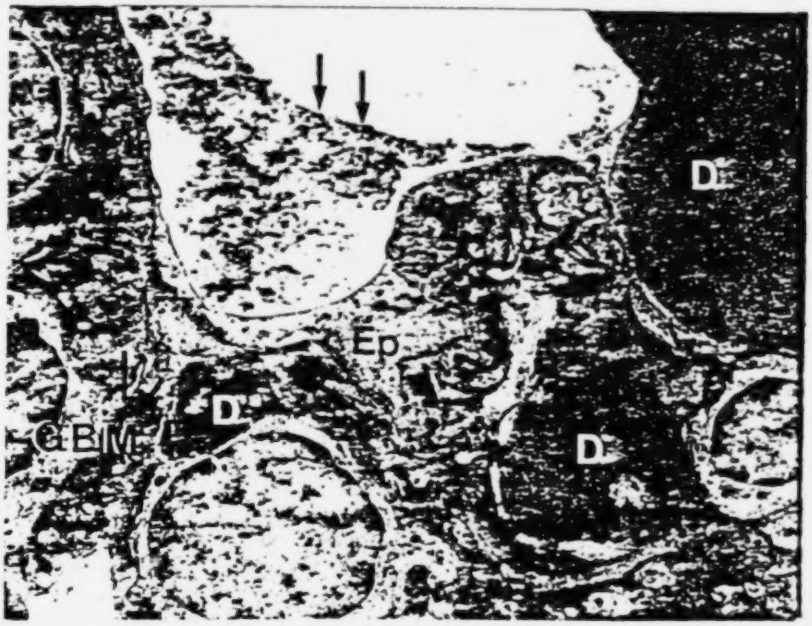

FIGURE 5. Ultrastructural demonstration of thickened glomerular basement membranes (GBM) electron dense deposits (D) and obliteration of epithelial (EP) foot process. Uranyl acetate, lead citrate, x 7,400.

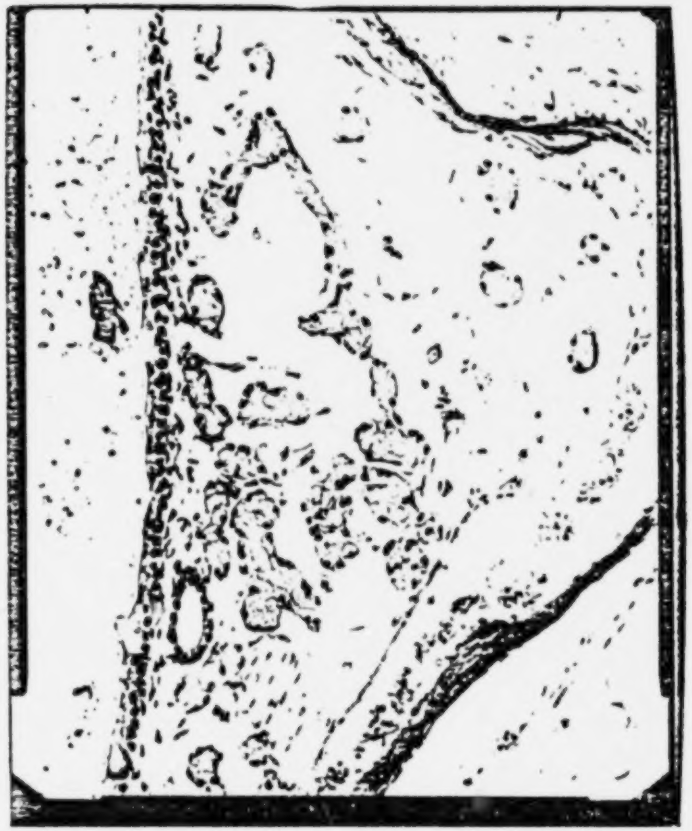

FIGURE 6. Amyloidosi in subepithelial layer of respiratory nasal region. (H & E, x 250).

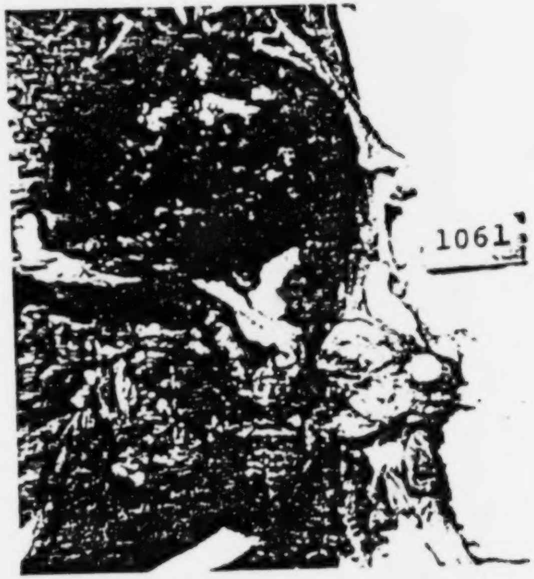

FIGURE 7. Histiocytic sarcoma arising within vagina and cervix and metastasizing to the liver.

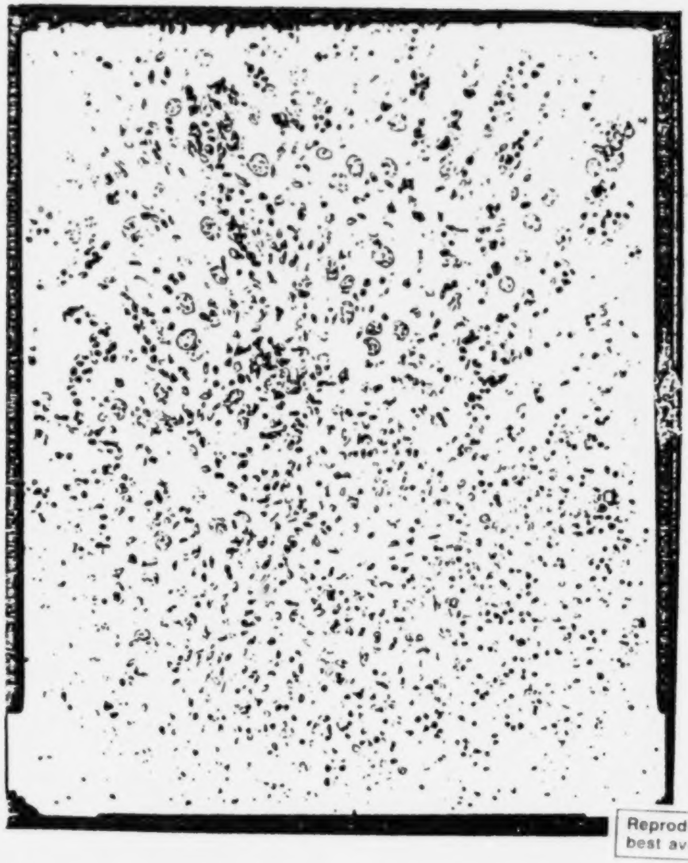

FIGURE 8. Histiocytic sarcoma in liver of SENCAR mouse. Alpha-1-antitrypsin was found in only a few tumor cels (arrows). ABC immunoperoxidase, hematoxylin, x 250.

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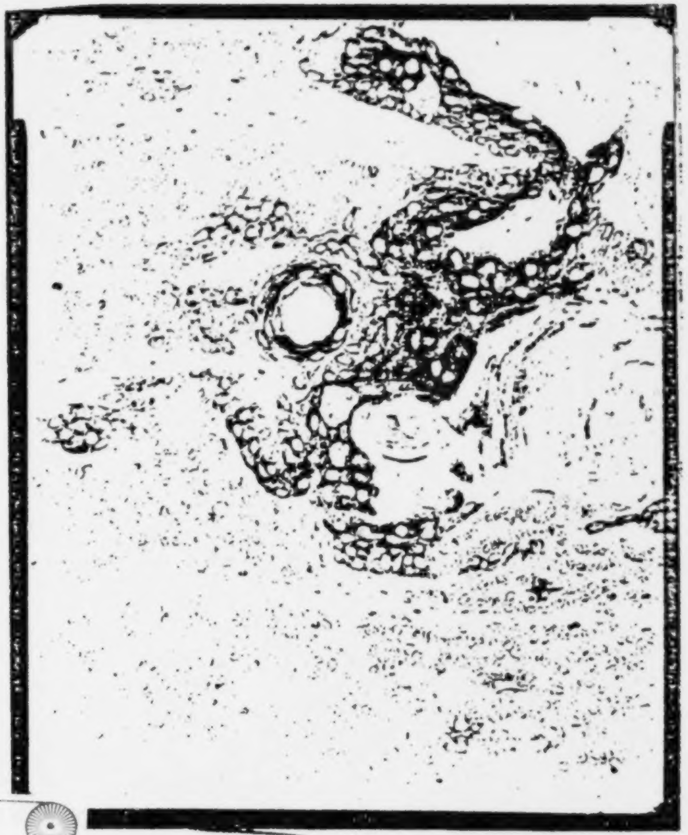

FIGURE 9. Keratin in areas of squamous metaplasia of a mammary adenoacanthoma (ABC immunoperoxidase, hematoxylin, x 250).

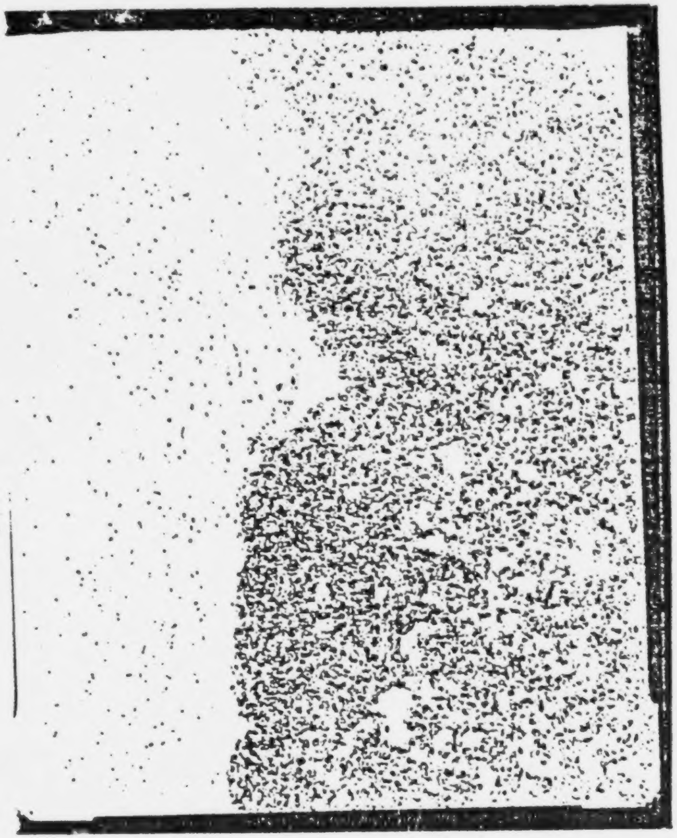

FIGURE 10. Prolactin in a pituitary tumor invading into brain parenchyma (ABC immunoperoxidase, hematoxylin, x 100).

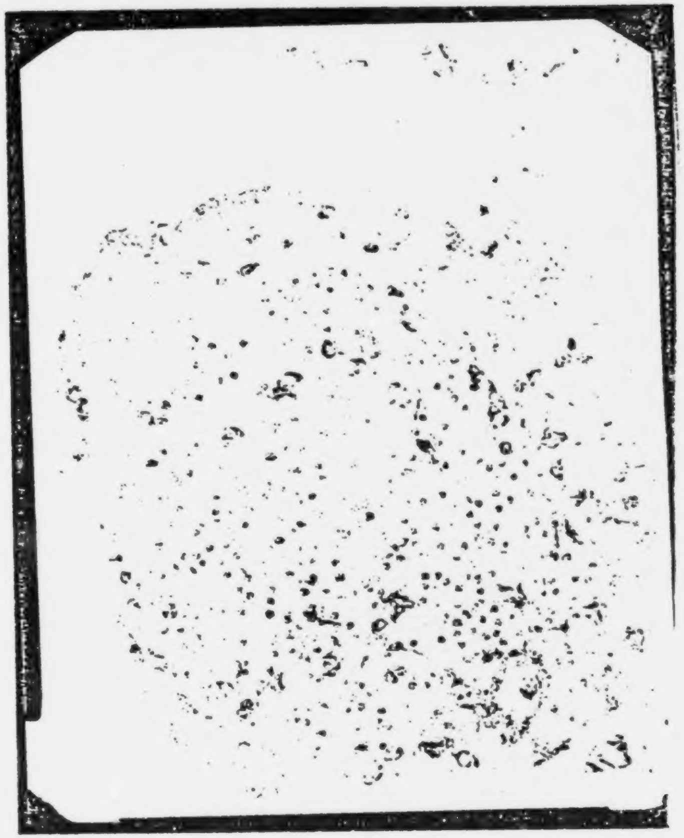

FIGURE 11. Glucagon in a hyperplastic pancreatic islet. The majority of the islet cells contained immunoractive insulin (ABC immunoperoxidase, hematoxylin, x 250).

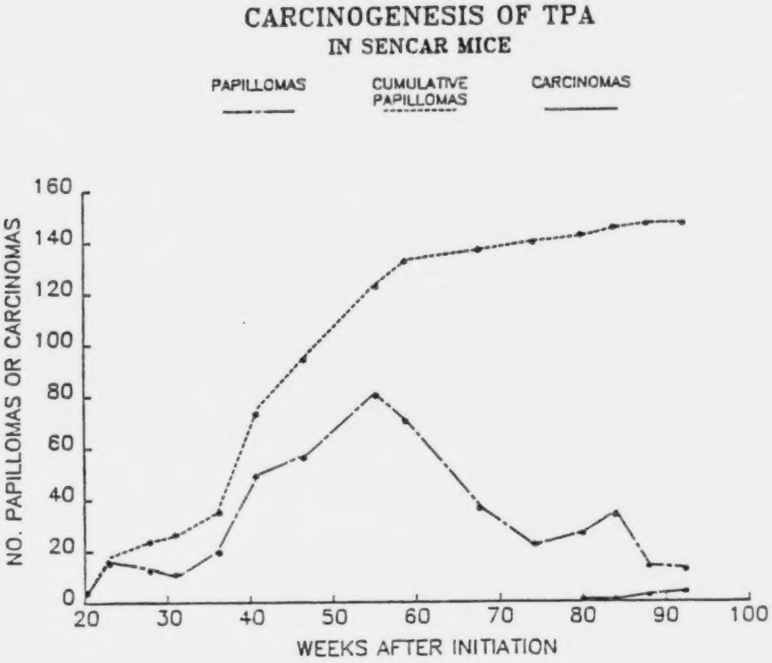

FIGURE 12. Skin papillomas and carcinomas seen clinically in female SENCAR mice receiving TPA, 1.25 μg once weekly, for 88 weeks. The decrease in papillomas was due primarily to regression of the lesions and also to mortality.

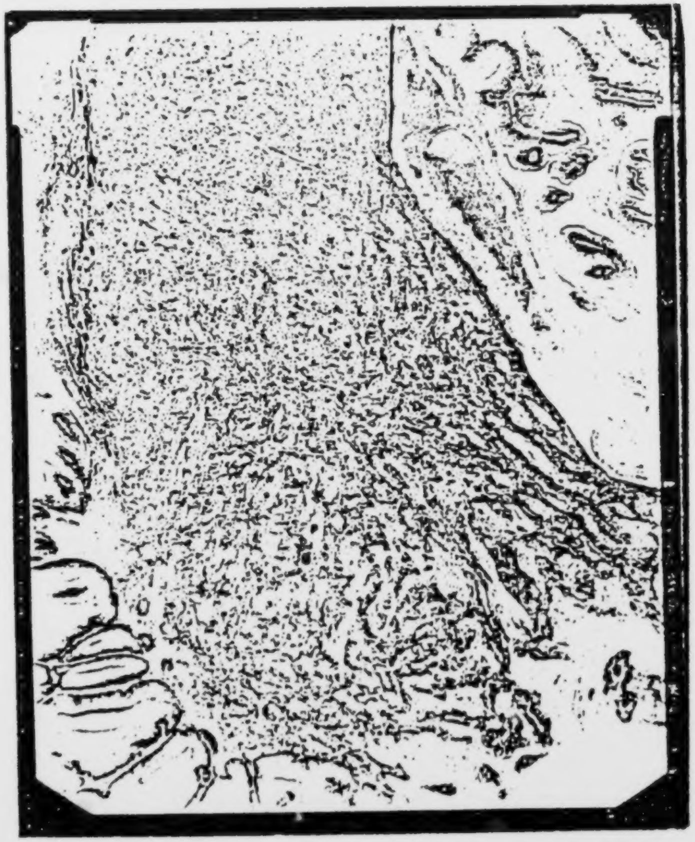

FIGURE 13. Poorly differentiated squamous cell carinoma arising within a papilloma of a SENCAR receiving TPA. (H & E, 40).

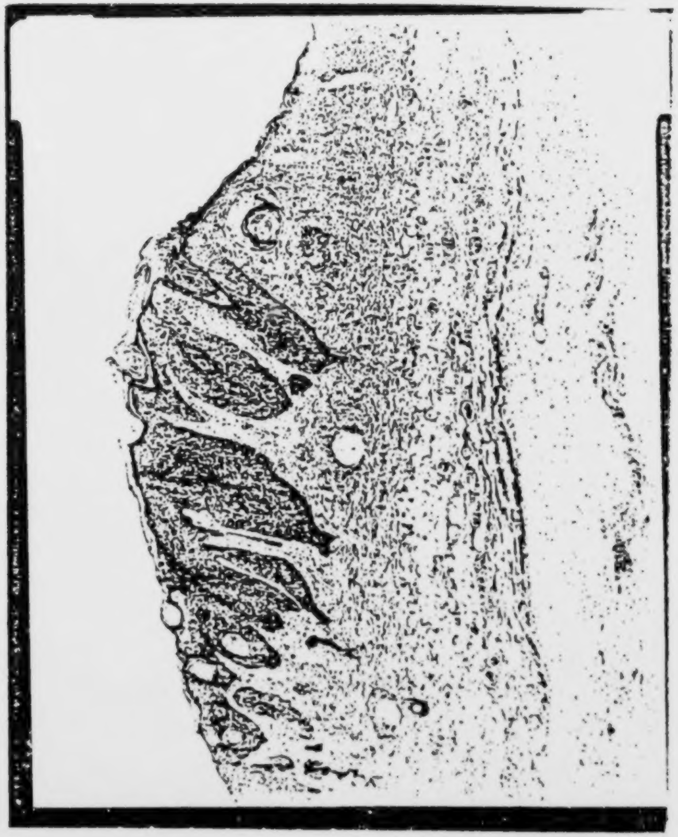

FIGURE 14. Ulcer, focal epidermal hyperplasia, and inflammatin in skin of mouse after 88 weeks of TPA exposure. (H & E, x 40).

MALIGNANT CONVERSION AND METASTASIS OF MOUSE SKIN TUMORS:
A Comparison of SENCAR and CD-1 Mice

by

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Key words: Malignant conversion, tumor progression, initiation, promotion, papillomas, carcinomas, SENCAR, metastasis

ABSTRACT

The progression of papillomas to squamous cell carcinomas (malignant conversion) was studied in the skin of SENCAR and Charles River CD-1 mice, utilizing a 3-stage treatment protocol. After initiation with 7,12-dimethylbenz[a]anthracene (Stage I) and limited promotion by 12-O-tetradecanoylphorbol-13-acetate (TPA) (Stage II), papilloma-bearing mice were treated (Stage III) with either tumor initiators, such as urethane, N-methyl-N'nitro-N-nitrosoguanidine (MNNG) or 4-nitroguinoline-N-oxide (4-NQO), the promoter TPA, or solvent (acetone). Similar final carcinoma yields were found in the mice treated in Stage III with TPA or acetone, although carcinomas developed earlier in the TPA-treated mice. In contrast, treatment with tumor initiators in Stage III increased both the rate of appearance and the final yield of carcinomas. Similar results were obtained in both SENCAR and CD-1 mice. A papilloma stage appears to be necessary for carcinoma development since elimination of TPA treatment in Stage II greatly reduced the incidence of both papillomas and carcinomas in both stocks of mice. The heterogeneity of papillomas with regard to progression to carcinomas is demonstrated by the low rate of conversion of TPA-dependent papillomas and the high rate of conversion of persistent papillomas in CD-1 mice. The carcinomas which develop using the 3-stage regimen vary in metastatic potential. In CD-1 mice, metastases to lymph nodes were similar in groups treated in Stage III with MNNG, urethane, 4-NQO, TPA or acetone, but treatment with urethane substantially increased metastases to the lung. In SENCAR mice, this effect of urethane was not observed, but lymph node and lung metastases appeared to be increased by Stage III treatment with MNNG.

INTRODUCTION

The initiation-promotion model of epidermal carcinogenesis in mice has provided the basis for many conceptual advances in the understanding of the biology of carcinogenesis (1-3). Initiation by a single treatment with a low dose of carcinogen such as 7,12-dimethylbenz[a]anthracene (DMBA) (Stage I) results in the permanent alteration of some epidermal cells (Table 1). In the absence of further treatment, these initiated cells do not develop into tumors, but the cellular changes are heritable. As a result of subsequent repeated exposure of initiated skin to promoting agents (Stage II), multiple benign papillomas develop. The promotion process is reversible; papillomas do not develop after insufficient promoter treatment or if the interval between treatments is prolonged. Many papillomas regress when the promoting stimulus is removed (4). Other papillomas progress irreversibly to carcinomas either spontaneously or as a result of further exposure to a genotoxic tumor initiator; this process has been termed malignant conversion (Stage III) (5). Continued treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) increases the rate of malignant conversions, but does not alter the final carcinoma incidence (5). The factors responsible for this conversion have only been studied recently, although Berenblum (6) recognized the conversion of benign papillomas to carcinomas as a specific stage nearly 45 years ago. Metastasis of skin squamous cell carcinomas to the lungs and lymph nodes occurs as a fourth stage in epidermal carcinogenesis.

The irreversibility of both initiation and malignant conversion and the genotoxic nature of the active agents suggests that these processes involve genetic alterations. The increased proliferation rate in promoter-treated epidermis and in papillomas may increase the probability of genetic changes necessary for the progression of papillomas to carcinomas (7,8). Three-stage carcinogenesis has been demonstrated only in SENCAR mice (5). In this report, results of further experiments with SENCAR mice are compared to those from similar experiments in Charles River CD-1 mice. Furthermore, rates and sites of metastasis of squamous cell carcimonas are compared in the two stocks of mice.

RESULTS AND DISCUSSION

Malignant conversion in SENCAR mice. In tumor induction experiments in Swiss mice, most squamous cell carcinomas progress from papillomas (9,10), but the rate of conversion is low. The factors necessary for this malignant conversion have not been defined. After DMBA initiation (Stage I) and limited TPA promotion (Stage II) in SENCAR mice, continued TPA treatment of these papilloma-bearing mice was compared to repeated exposure to an initiator for the effect on carcinoma development (Stage III). Exposure to the promoter TPA in Stage III does not influence the final carcinoma yield, but the treatment of papilloma-bearing mice with genotoxic tumor initiators markedly accelerates and enhances malignant conversion. The carcinoma incidence was increased by Stage III treatments with 4-nitroquinoline-N-oxide (4-NQO), N-methyl-N'nitro-N-nitrosoguanidine (MNNG) and urethane for 30 weeks (5). Control groups of SENCAR mice treated only with the active Stage III agents showed that urethane induced no tumors, 4-NQO induced few papillomas and no carcinomas, while MNNG was moderately active as a complete carcinogen (5).

When this experiment was repeated, Stage III 4-NQO or urethane treatment of papilloma-bearing mice for 30 weeks again induced carcinomas in more than half the mice within 30 weeks (Fig. 1). Exposure to acetone or TPA in Stage III resulted in fewer carcinomas with a longer latent period (see Fig. 5), as reported earlier (5). In control groups treated with acetone rather than TPA in Stage II, Stage III treatment with 4-NQO or urethane induced few carcinomas (Fig. 1). These carcinomas appeared 20 weeks or more later than those in mice treated with TPA in Stage II. In contrast, with MNNG in Stage III, a high carcinoma yield was obtained even when TPA promotion in Stage II was omitted (data not shown).

Thus, MNNG is not useful as a Stage III agent when given for 30 weeks. Fewer exposures to MNNG, from one to 10 weeks, are currently being tested for Stage III activity.

Malignant conversion in Charles River CD-1 mice. In order to verify the 3-stage carcinogenesis results in mice other than SENCARs, a similar experiment was performed in Charles River CD-1 mice. In CD-1 mice initiated with DMBA (Stage I) and promoted with TPA for 12 weeks (Stage II), many papillomas regressed after TPA exposure was terminated (Fig. 2). If TPA was continued, the papilloma incidence continued to rise until Week 28. However, the final carcinoma yield was essentially equal in groups receiving continuous TPA or just acetone solvent in Stage III. Since the number of papillomas remaining at the time carcinomas were first seen was markedly reduced in the acetone group, TPA-dependent papillomas are at very low risk for carcinoma formation while persistent papillomas (those which remain after termination of TPA treatment) are at high risk. The maximum papilloma incidence at 16 weeks was reduced 30% by treatment with 4-NQO in Stage III, but the papilloma regression rate was unaffected by urethane or 4-NQO compared to that in acetone-treated mice (Fig. 2). Either 4-NQO or urethane in Stage III clearly increased both the rate of carcinoma appearance and the final carcinoma yield relative to TPA or acetone treatment (Fig. 2). The low carcinoma yield in control groups not exposed to TPA in Stage II confirms the requirement for a papilloma stage prior to carcinoma formation (Fig. 3). Stage I initiation followed by solvent treatment in Stage II and urethane or 4-NQO in Stage III produced few papillomas (3 with urethane and 31 with 4-NQO per 40 mouse group) and almost no malignancies (Fig. 3). However, repeated MNNG given to DMBA-initiated or uninitiated CD-1 mice induced carcinomas in over half of the mice (Fig. 4), as we observed in SENCAR mice.

The percent conversion of papillomas to carcinomas was calculated at Week 60 based on the maximum number of papillomas which developed in each group shown in Fig. 2. With continued TPA treatment, a 2.5% malignant conversion rate was found, compared to 3.7% in the acetone control group. The final percent conversion was increased to 5.5% with urethane and to 7.9% with 4-NQO. These percentages are approximately twice those calculated in SENCAR mice (5) since the papilloma incidence is about half that found in SENCARs and the carcinoma incidence is comparable. The difference between mice treated with acetone or TPA and mice treated with genotoxic agents in Stage III was much more striking at earlier times. At 40 weeks, the percent conversion was 0.75% with acetone, 1.2% with TPA, 4.2% with urethane and 6.3% with 4-NQO. Thus, genotoxic agents in Stage III not only increase the number of carcinomas but accelerate their appearance.

Malignant conversion in SENCAR or CD-1 mice treated with TPA in Stage III. In contrast to CD-1 mice, continued TPA treatment of initiated SENCAR mice after an optimal course of TPA treatment did not result in a maintenance of a high papilloma incidence (Fig. 5). Papillomas regressed, whether mice were exposed to continued TPA or acetone solvent. The mechanism responsible for this apparent absence of TPA-dependent papillomas in SENCAR mice is unclear. As with CD-1 mice, however, the final carcinoma yield was the same with TPA or solvent, although the latent period may be somewhat shorter in the continuous TPA group.

Metastasis of squamous cell carcinomas. In the 3-stage carcinogenesis experiment in CD-1 mice described in Figures 2-4, complete autopsies were performed on all animals and possible metastatic lesions were examined histologically. After DMBA initiation, 12 weeks of TPA promotion and either continued TPA or acetone treatment for a further 40 weeks, about 20% of the mice with 1 or more carcinomas also developed metastatic carcinomas (Table 2). With MNNG in Stage III, the overall metastasis rate increased to 30%, but with 4-NQO, a metastasis rate of only 15% was found. With acetone, TPA, MNNG or 4-NQO, a total of 14 metastases were seen in lymph nodes with only 4 in the lungs. Both the pattern and frequency of metastasis were altered in the mice exposed to urethane. Metastases were seen in one-third of the carcinoma-bearing mice, with 3 in lymph nodes and 6 in lungs. The lung squamous cell carcinomas seen after Stage III urethane exposure were not primary urethane-induced lesions since identical urethane treatment of uninitiated mice or initiated mice not treated with TPA in Stage II produced no squamous cell carcinomas of the skin or lung. Primary lung adenomas were observed in about half the mice in these 2 groups as well as in the group which developed metastatic lung tumors.

The overall rate of metastasis appeared to be slightly lower in SENCAR mice treated in Stage III with acetone (Table 2). A group treated with TPA in Stage III was not included in this experiment, so no data is presented on metastases in SENCAR mice treated long-term with TPA. In contrast to CD-1 mice, Stage III exposure to urethane did not affect the number of mice with lung metastases. While 4-NQO increased the metastatic rate only slightly, MNNG in Stage III more than doubled the overall rate of metastasis.

Table 1. Multistage Epidermal Carcinogenesis

Stage	End Point
I. Initiation	Initiated cells
II. Promotion (Stages 1 and 2)	Papillomas
III. Malignant Conversion	Squamous cell carcinomas
IV. Metastasis	Metastatic squamous cell carcinomas

Table 2. Frequency and distribution of metastases in mice bearing skin squamous cell carcinomas.

Stage III Treatment	Percent of carcinoma-bearing mice with metastasis					
	Total (one or more sites)		Lymph Node		Lung	
	CD-1	SENCAR	CD-1	SENCAR	CD-1	SENCAR
Acetone	16.7	11.1	16.7	5.6	11.1	11.1
TPA	23.1	--	23.1	--	0	--
MNNG	30.0	26.3	25.0	15.8	5.0	15.8
Urethane	33.3	11.1	12.5	0	25.0	11.1
4-NQO	15.0	19.0	15.0	9.5	5.0	9.5

Groups of 40 female CD-1 mice were initiated with 50 μg DMBA (Stage I) and promoted with 12 weekly applications of 10 μg TPA (Stage II). In SENCAR mice, groups of 30 females were initiated by 20 μg DMBA and promoted with 10 weekly applications of 2 μg TPA. The indicated Stage III treatments once weekly from Week 13-52 in CD-1 mice and Week 11-40 in SENCAR mice were as follows: acetone, 0.2 ml; TPA, 10 μg; MNNG, 120 μg; urethane, 20 mg i.p.; 4-NQO, 250 μg. Some animals developed metastases in both lymph nodes and lungs. Between 13 and 24 carcinoma-bearing mice were examined in each group. Squamous cell carcinomas in the lungs or lymph nodes were observed only in mice which bore skin squamous cell carcinomas.

ACKNOWLEDGMENTS

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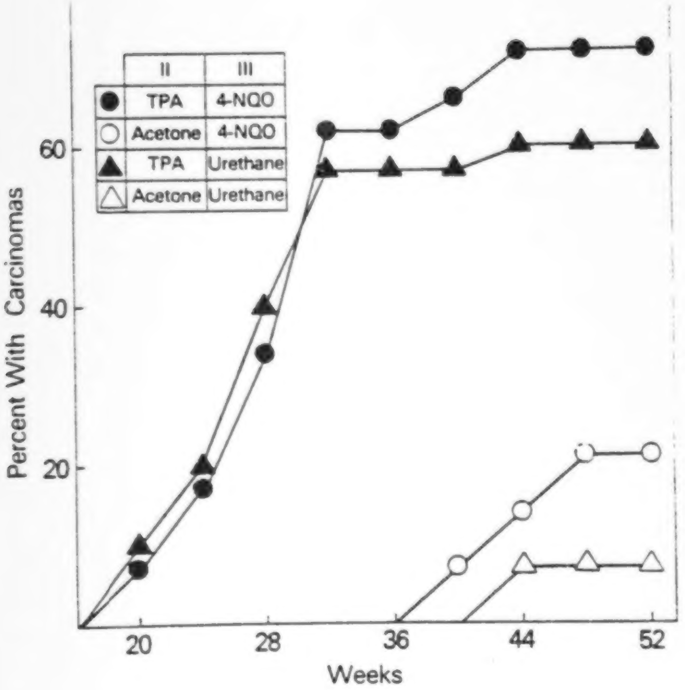

Figure 1. Carcinoma incidence in 3-stage carcinogenesis in SENCAR mice. Groups of 30 female SENCAR mice were initiated with 20 µg DMBA (Stage I) and treated with 10 weekly applications of either TPA (solid figures) (2.0 µg/0.2 ml acetone) or acetone (open figures) in Stage II. Once weekly treatments with urethane (triangles) (20 mg i.p.) or 4-NQO (circles) (250 µg/0.2 ml acetone) continued from Week 11-40 in Stage III. Suspected carcinomas were scored bi-weekly and were verified by histological examination.

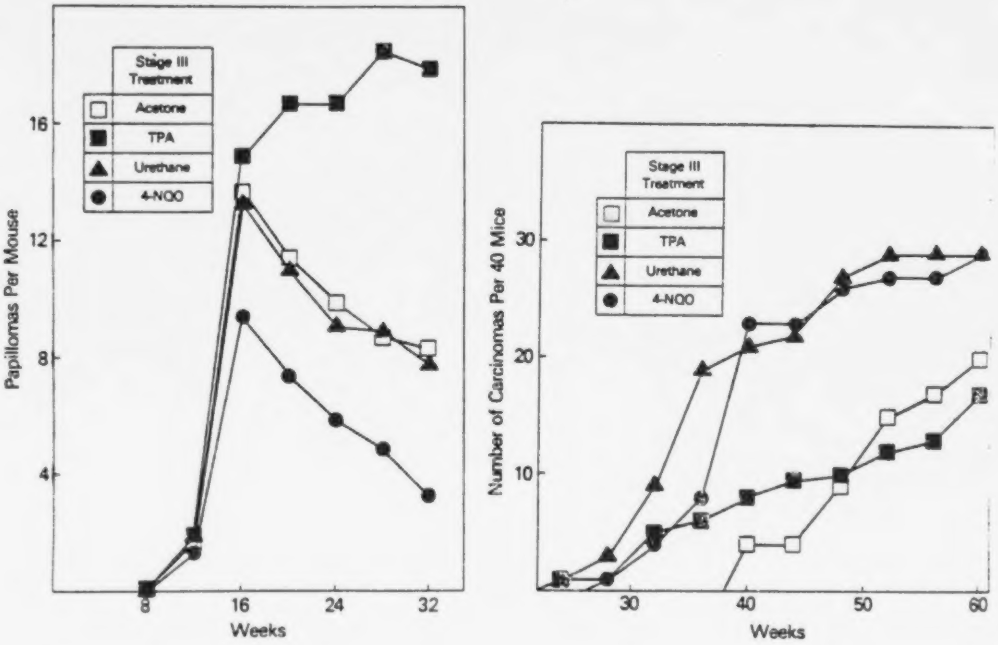

Figure 2. Papilloma and carcinoma incidence in a 3-stage carcinogenesis experiment in CD-1 mice. Groups of 40 female CD-1 mice were initiated with 50 µg DMBA (Stage I) and promoted with 12 weekly applications of 10 µg TPA (Stage II). The indicated Stage III treatments once weekly from Week 13-52 were as follows: acetone, 0.2 ml (□); TPA, 10 µg (■); urethane, 20 mg i.p. (▲); 4-NQO, 250 µg (●).

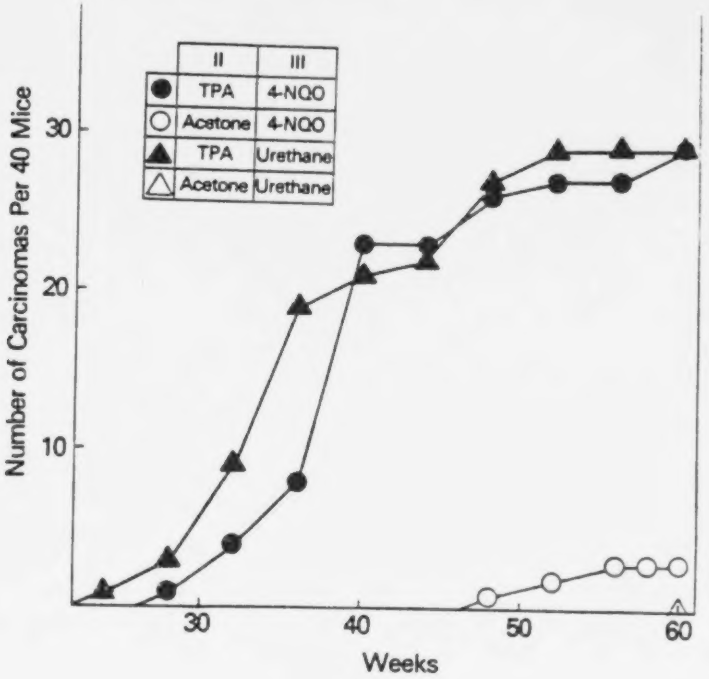

Figure 3. Carcinoma incidence in 3-stage carcinogenesis in CD-1 mice. In the experiment described in Fig. 2, treatment with TPA (solid figures) is compared to treatment with acetone (open figures) in Stage II.

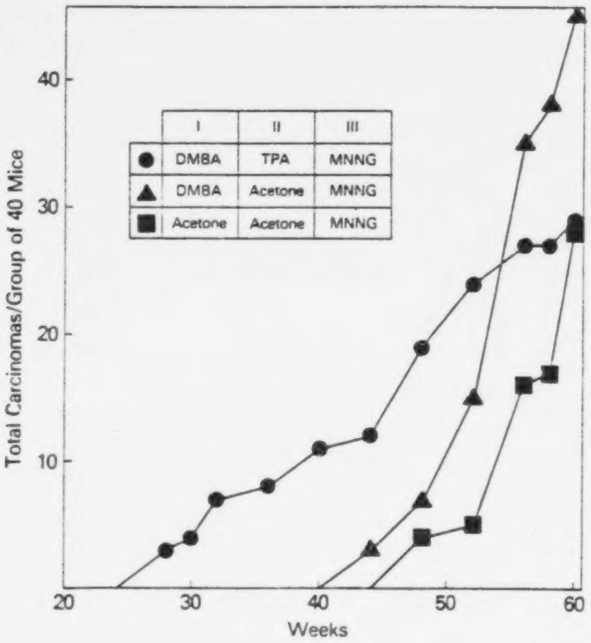

Figure 4. Carcinoma incidence in CD-1 mice treated with MNNG in Stage III. Mice were treated with DMBA (●,▲) or acetone (■) solvent in Stage I. In Stage II, mice received either TPA (10 μg/0.2 ml acetone once weekly for 12 weeks) (●) or 0.2 ml acetone once weekly for 12 weeks (▲,■). All 3 groups were treated with MNNG (120 μg/0.2 ml acetone) in Stage III.

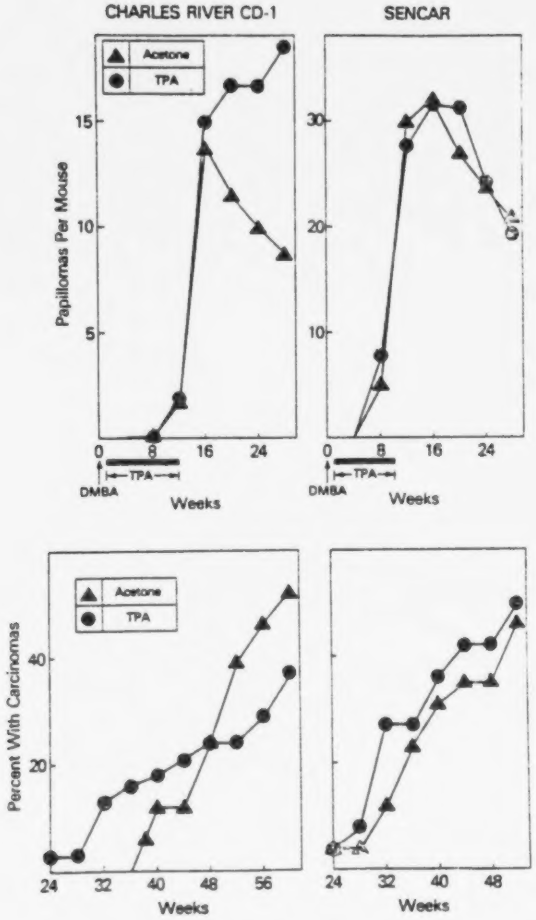

Figure 5. Papilloma and carcinoma incidence in papilloma-bearing mice treated with TPA or acetone solvent: comparison of CD-1 and SENCAR mice. Charles River CD-1 mice were initiated with 50 µg DMBA and promoted with 12 weekly applications of 10 µg TPA. Beginning at Week 13, mice were treated once per week with either 0.2 ml acetone (▲) or 10 µg TPA/0.2 ml acetone (●). Similarly, papillomas were induced in SENCAR mice by initiation with 20 µg DMBA and promotion by 10 weekly applications of 2.5 µg TPA. Beginning at Week 11, mice were treated weekly with either 0.2 ml acetone (▲) or 2.5 µg TPA/0.2 ml acetone (●).

EFFECTS OF CHRONIC TOPICAL APPLICATION OF 12-0-TETRADECANOYLPHORBOL-13-ACETATE ON THE SKIN AND INTERNAL ORGANS OF SENCAR MICE

by

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Key Words: epidermal hyperplasia, amyloidosis, nephritis, reative lymphoadenopathy, tumor promoter, 12-O-tetradecanoylphorbol-13-acetate

Abstract

Repetitive topical applications of 2 μg TPA twice weekly for 37 to 52 weeks induced a sustained epidermal hyperplasia, hyperplasia of hair follicles and increased dermal cellularity in SENCAR mice. In addition, after 52 weeks of protracted promoter treatment most animals developed generalized amyloidosis involving liver and spleen, as well as, interstitial nephritis. Severe pyelonephritis and papillary necrosis were also frequently seen. Reactive lymphoid hyperplasia was also a frequent finding. Chronic administration of TPA is not an inocuous treatment affecting only the interfollicular epidermis. The general effects of the promoter or the animals, probably through impairment of the immune system, decreased markedly their longevity.

Introduction

The *in vivo* effects of tumor promoters, especially those produced by topical application of a single dose of 12-0-tetradecanoyl-phorbol-13-acetate (TPA), have been extensively described (1-7). The study of the effects of multiple repetitive applications of TPA on initiated skin, the only schedule of administration eventuating in significant tumor production, has been partially studied by a few investigators (8-10). Even less is known about the systemic effects of TPA when animals are subjected to protracted promoter treatments.

In two separate studies we have analyzed the effects of chronic topical applications of 2 μg TPA on the uninitiated skin of SENCAR mice and on other organs during 37 to 52 weeks of repetitive twice-weekly treatments.

Effects of 74 TPA applications on mouse skin

Epidermal hyperplasia: The effects of repetitive treatments of TPA on the mouse skin were studied using 3HTdr pulse labeling and morphometric techniques. A sustained marked epidermal hyperplasia (4-5 x normal values) (Fig. 1 and 2) was a constant feature during chronic exposure to TPA. Only 5% of the animals developed papillomas (maximum 2 papillomas/animal), and no carcinomas. The percentage of dark cells, the labeling index of the basal layer, and the epidermal thickness during chronic TPA treatment are shown in Fig. 1a and b. The pattern of the curves is similar, both show an early peak, after 4 treatments, descending slightly thereafter. The epidermal thickness values correlated well with number of nucleated cell layers (data not shown) indicating that the increase seen in the first portion of the curve was not due to edema only. Although an early peak was not seen, the number of hair follicles per length of epidermal surface increased and also reached equilibrium after the eighth TPA application.

Cellular Changes in the Dermis

The total dermal thickness increased immediately after the beginning of TPA application. This initial increase of approximately 50% was evident after 4 TPA treatments (Fig. 3a) and was attributed to edema and acute inflammation. Later on, additional gradual increase was observed. This increase of dermal thickness observed after 40 TPA applications was due to the production of a fibrotic dermis. The total dermal cellularity increased with the number of treatments, reaching a plateau after the eighth application (Fig. 3b). The relative proportion of the different cellular types changed during the course of the treatment. After the first TPA application, the two predominant cellular types were the polymorphonuclear leukocytes and the fibroblasts. The number of polymorphonuclear leukocytes dropped dramatically during the first weeks of treatment. The lymphocytes increased from 2.4% in controls to 15.7% on the 8th application. The macrophages, also involved in the chronic inflammatory reaction, increased slowly during treatment. The number of mast cells was always higher than in the control tissues (Fig. 3c). During the later part of the chronic treatment an increase of fibroblasts and capillary vessels was observed.

Effects of 104 TPA applications on mouse skin

Figure 4 shows the survival of Sencar mice treated with doses of 1 and 2 μg. After 12 months it became obvious that topical treatment with this tumor promoter, especially with doses of 2 μg was markedly lethal, approximately equivalent to a LD 50.

The general characteristics of the cutaneous alterations were the the same as after 80 treatments. However, some additional changes were observed. In some animals cystic dilatations and atrophic changes were observed in the hair follicles, eventuating in a few cases in the formation of epidermoid cysts of 1 to 10 mm diameter. In addition, and related to the same phenomenon, we could observe the presence of multiple keratinic granulomas in the superficial dermis of six animals (Fig. 5). In 7 animals out of 75 marked hyaline and or amyloid changes of the papillary dermis could be seen. Cutaneous ulcers, sometimes exhibiting exuberant granulation tissue, were also observed. In these cases, as well as in a few others without ulceration, regional lymph nodes showed changes of interfollicular and/or follicular hyperplasia (Table I).

Effects of 104 TPA applications on other organs

Table II summarizes the general findings after 52 weeks of treatment.

Generalized amyloidosis was the most frequently encountered alteration (62% of animals), the liver and spleen were always affected (Fig. 6); kidney was frequently involved, and adrenal, skin and intestine contained occassional amyloid deposits. Chronic focal interstitial nephritis was very frequently seen (60%) and in some cases exhibited a clear picture of chronic pyelonephritis. In several cases a nephropathy involving all kidney elements, including glomerular fibrosis and interstitial chronic inflammation, could be seen. Papillary necrosis (Fig. 7) accompanied, very frequently, the most advanced cases of nephritis and/or renal amyloidois. Systemic lymphoadenopathies, mainly of inguinal, axillary, mesenteric and mediastinal nodes were observed in many animals. In most cases, the normal lymph node architecture was conserved and only a reactional (hyperplastic) chronic lymphadenitis could be recognized. However, in several cases, especially in those with cutaneous erosions and/or ulcers, a more dramatic picture with total or partial effacement of the node architecture was observed. These alterations, consistent with the diagnosis of dermatopathic lymphadenopathy, were enlargement of paracortical areas due to the presence of a histiocytes and activated lymphocytes.

In 37% of the animals chronic adenitis with marked proliferation of endothelial cells and capillary vessels, as well as the presence of large, sometimes atypical, lymphocytes, plasma cells and immunoblasts was seen (Fig. 8). This histological picture is similar to angioimmunoblastic lympadenopathy, a human disease characterized by hyperproliferation of B-cells. In two other cases we have seen a similar picture, including cell destruction, presence of polymorphonuclear leukocytes, actively phagocytic macrophages and mitosis. Frequently these alterations were accompanied with intranodal foci of extramedullary hematopoyesis. Although a few benign tumors and one squamous carcinoma of the skin were seen after 104 TPA applications, this was not significantly different from the spontaneous incidence of malignant skin tumors found in a control group (Table III). Similarly, no differences were found in the incidence of neoplasias of the lung and hematopoyetic organs.

Discussion

Except for the cases which presented with ulcers or erosions, repetitive treatment of skin with promoting doses of TPA produces a steady state epidermal hyperplasia with an increased dermal cellularity. Most of dermal cells were fibroblasts and endothelial cells, resulting in a very dense and hypervascularized dermis. It is interesting to note that most authors have focused on the hyperplasiogenic effects of TPA on the epidermis and have overlooked cell proliferation in the dermis. Similarly, with the exception of one group of investigators (9), the effects of promoters on hair follicles were not studied. We were able to confirm, with a different technique, the induction of new hair follicles described in wholemounts of TPA treated skin (9). From these statements it is easy to conclude that the interfollicular epidermis is not the only target of TPA. The hair follicles, as well as the dermal components, are fully affected by the promoter treatment. Hair follicles have been singled-out very frequently as the origin of cutaneous tumors, including papillomas. The effects of the mesenchyme on the differentiation of the epidermis, as well as the possible role of hair follicles, are important facts that should be considered in the pathogenesis of tumors induced by two-stage carcinogenesis in Sencar mice.

Skin ulcers were seen in many animals after 104 TPA applications and were always accompanied by regional, and sometimes, distant reactive lymph node hyperplasia. This lymph node reaction, although especially evident in these cases, was also seen in most animals without cutaneous ulcers. Table 3 shows that the majority of animals presented with lymphadenopathies but only the males exhibited skin ulcers, probably related to biting and fighting. Consequently, it is more than feasible that the reactive lymphadenitis seen in most of these animals is not due to secondary inflammation but to the effect of TPA. The histopathology of lymph node lesions was not uniform. Although all showed components of chronic reactive lymphadenitis, they also included foci of extramedulary myelopoyesis, angioblastic proliferation, as well as the presence of histiocytes and atypical lymphocytes. Some of these alterations were compatible with lesions observed in human immune-system dysfunctions, such as those seen in acquired immunodeficiency syndrome (11) dermatopathic lymphoadenopathy (12-13), and angioimmunoblastic lymphadenopathy (14-15). TPA has been shown to modify the response of murine T lymphocytes and macrophages (16), and to induce the proliferation of the granulocyte macrophage precursor cells (17). Consequently, the lymph node alterations seen in our animals might indicate a direct effect of TPA on lymph node architecture and function which warrants further study. Another probable evidence of a immunological disturbance is the generalized amyloidosis seen in the liver, spleen and kidney of most animals and occassionally also observed in the skin, intestines and other organs. Although the distribution of the amyloid deposits coincides with the secondary (AA) type (18), an accelerated form of systemic senile anyloidosis (AS/SAM) as described by Takeda and colleagues (19) cannot be excluded. This possibility is enhanced by the fact that untreated 18 to 30 month old SENCAR mice are frequently affected by generalized amyloidosis. It is also worthwhile to note that papillary necrosis seen in 35% of TPA treated animals was described in another mouse strain with renal amyloidosis (20). Interstitial nephritis and chronic pyelonephritis were seen in most animals after prolonged TPA treatment. Whether this is primary, and directly related to an eventual nephrotoxic property of TPA, or, secondary as is more probably the case, remains to be investigated.

Although some authors have described the production of a significant number of skin tumors in mice treated with TPA alone (21,22), we have not been able to find differences between the incidence of carcinomas of untreated and TPA treated SENCAR mice. Although TPA increased the level of papillomas seen in SENCAR mice, the papilloma yield was 0.23 papillomas per mouse in females and 0.04 papillomas per mouse in males. It has to be pointed out that the tumor yield in initiated animals is much higher, i.e. 7 papillomas/mouse at 15 weeks (23).

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Table I

PERCENT OF ANIMALS WITH SKIN ULCERS AND REACTIVE LYMPHADENOPATHIES

	MALES		FEMALES	
	DIED BEFORE 1 YEAR	KILLED AFTER 104 TPA APPLICATIONS	DIED BEFORE 1 YEAR	KILLED AFTER 104 TPA APPLICATIONS
LYMPHADENOPATHIES	45	70	57	71
SKIN ULCERS	45	18	28	3

Table II

Pathology of Animals Killed After 104 TPA Applications

	Males	Females
Liver and Spleen Amyloidosis	63%	61%
Interstitial Nephritis and Pyelonephritis	67%	54%
Papillary Necrosis	34%	35%
Adenopathies (Reactive hyperplasia)	40%	35%
Skin Papillomas	4%	19%
Skin Carcinomas	4%	0%
Lung Adenomas	4%	4%

Table III

SKIN TUMORS IN SENCAR MICE

		NUMBER OF ANIMALS WITH PAPILLOMAS	PAPILLOMAS/ MOUSE	NUMBER OF ANIMALS WITH CARCINOMAS	CARCINOMAS/ MOUSE
NO TREATMENT	MALE	0/127	0	4/127	0.03
	FEMALE	1/220	0.004	0/220	0
104 TPA (2 μg) TREATMENTS	MALE	1/27	0.04	1/27	0.04
	FEMALE	5/26	0.23	0/26	0

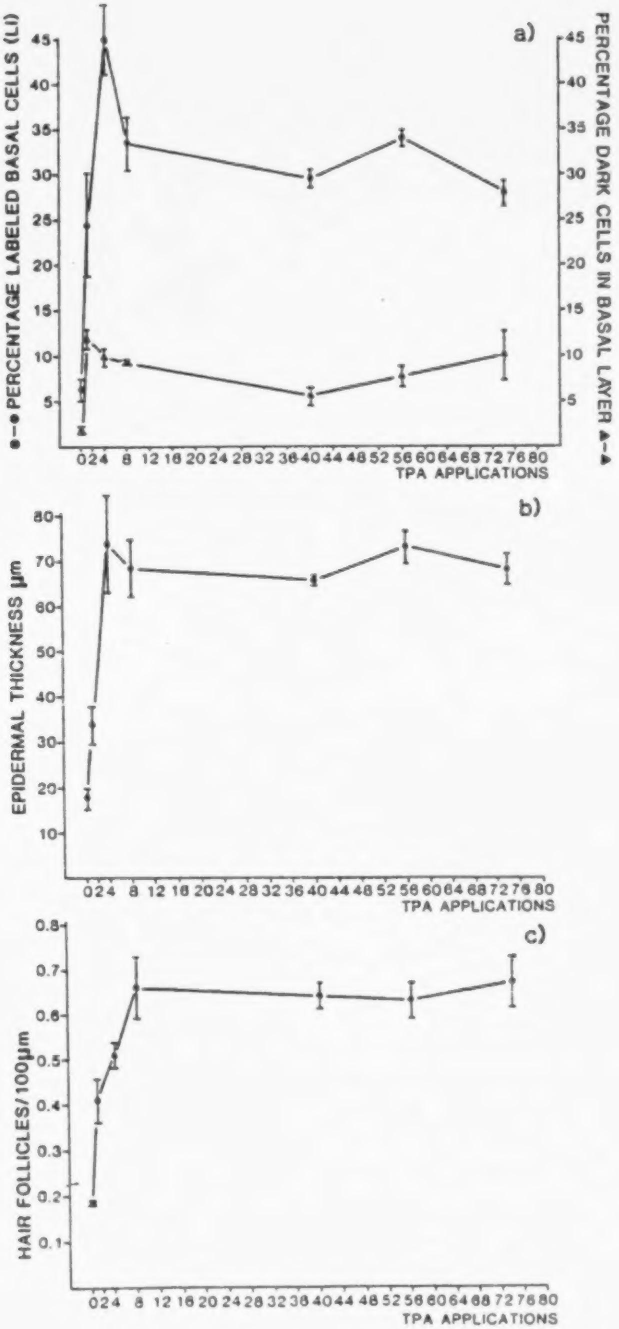

Figure 1: Morphometric data of epithelial structures of the skin.

a) percentage of labeled basal cells and percentage of dark basal keratinocytes; b) epidermal thickness; c) number of hair follicles. From Reference 10 (with permission).

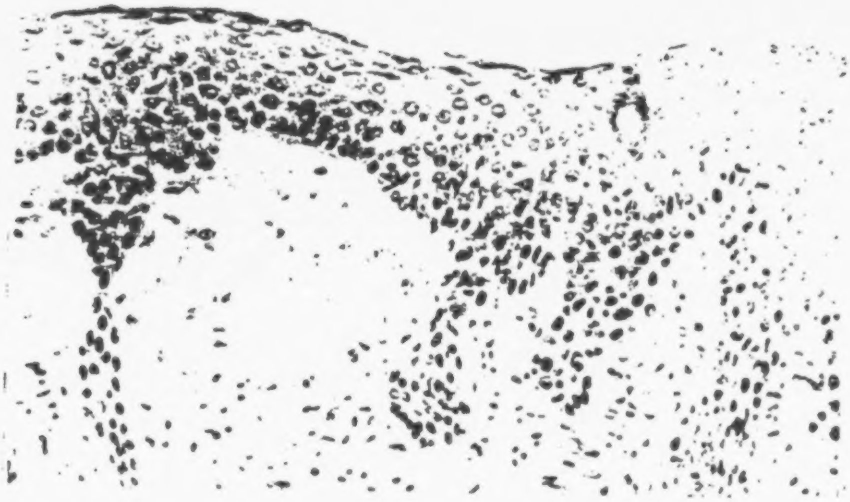

Figure 2: Epidermal hyperplasia after 74 applications of 2 μg TPA.

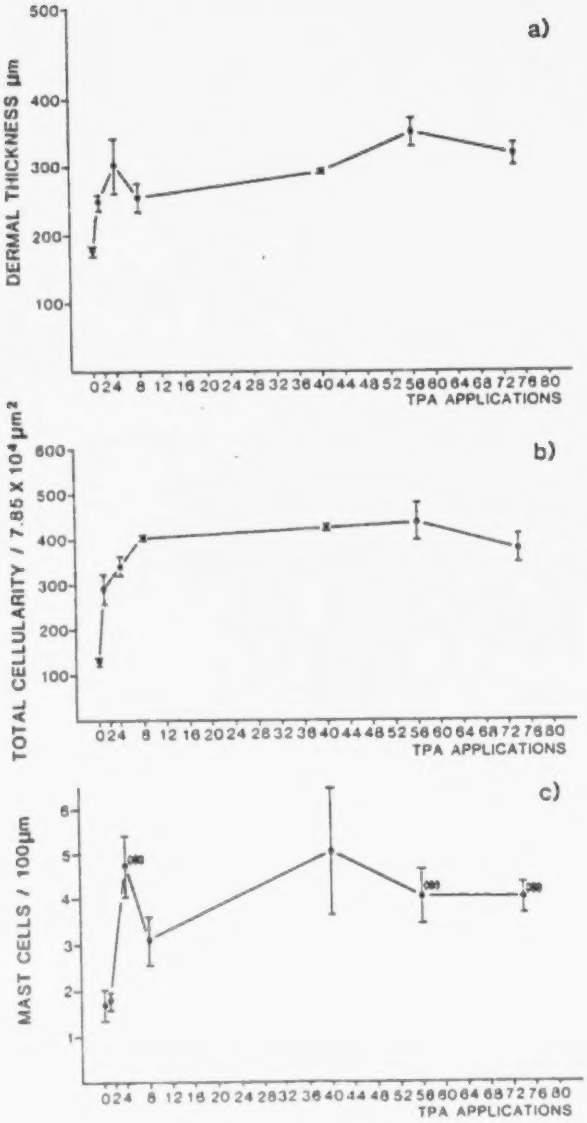

Figure 3: Morphometric evaluation of TPA induced dermal changes.

a) dermal thickness form basement membrane to the subcutaneous adipose tissue; b) total dermal cellularity per microscopic field; c) number of mast-cells per length of epidermal surface. From Reference 10 (with permission).

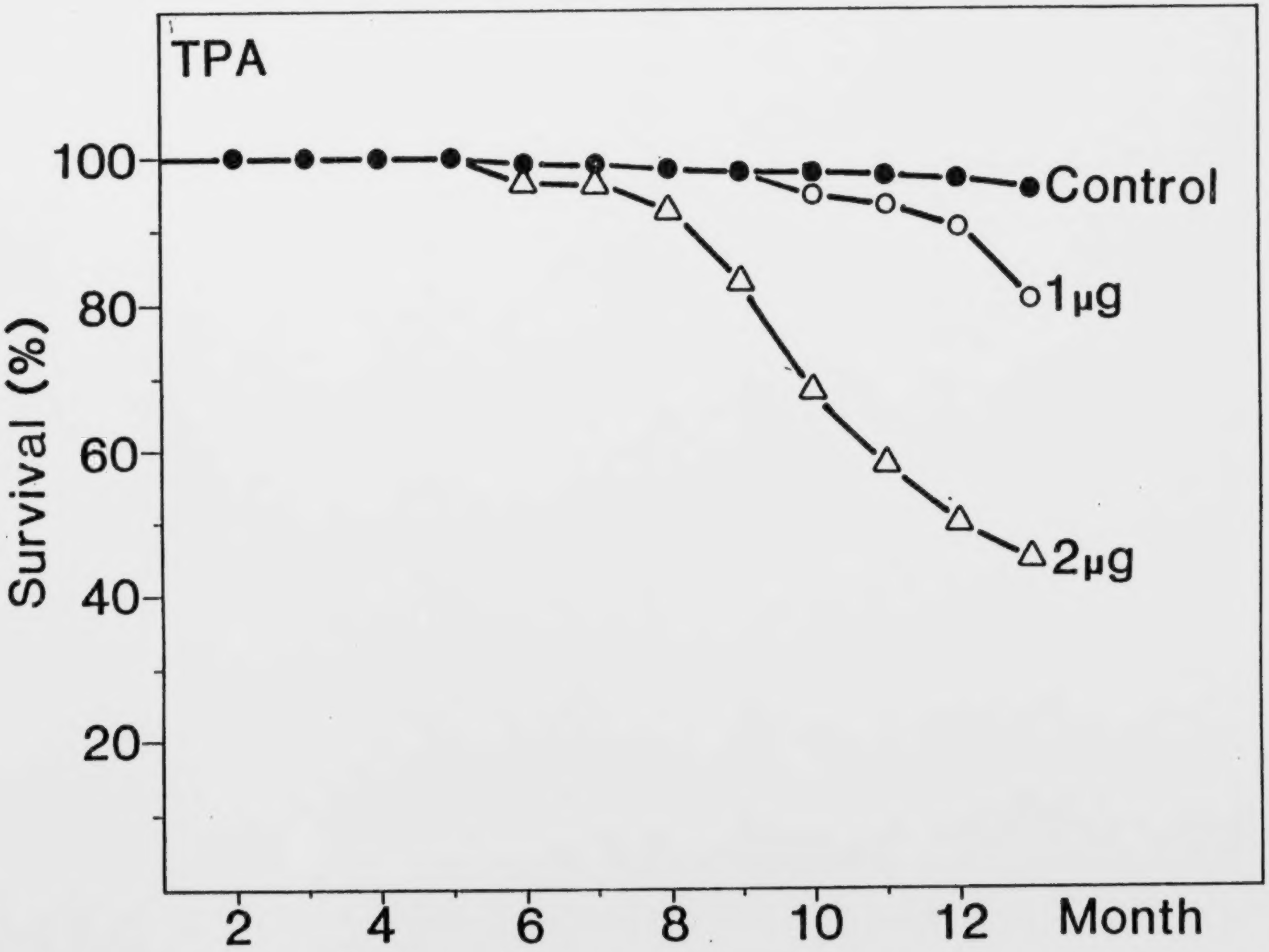

Figure 4: Survival curves of SENCAR mice treated topically with TPA.

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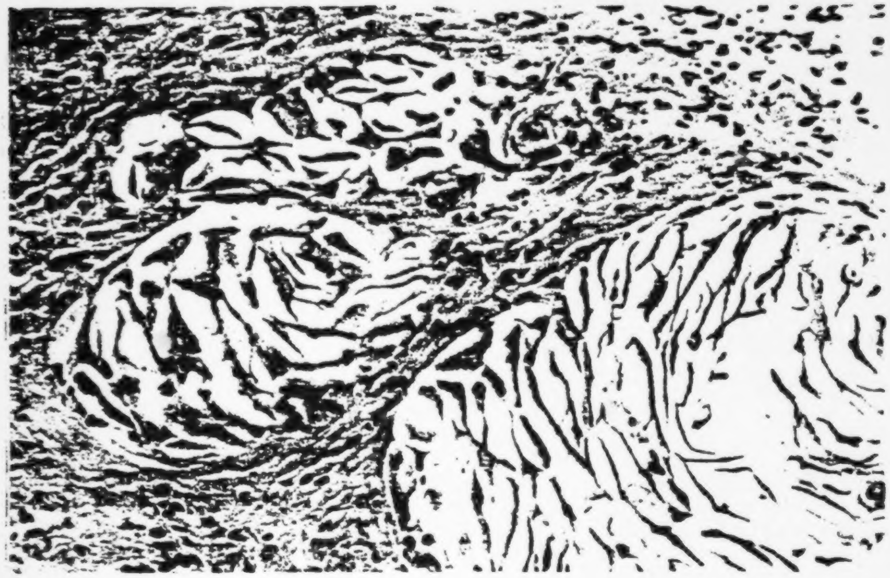

Figure 5: Dermal keratinic granuloma with giant cell reaction around horny squames, after 104 applications of 2 μg TPA.

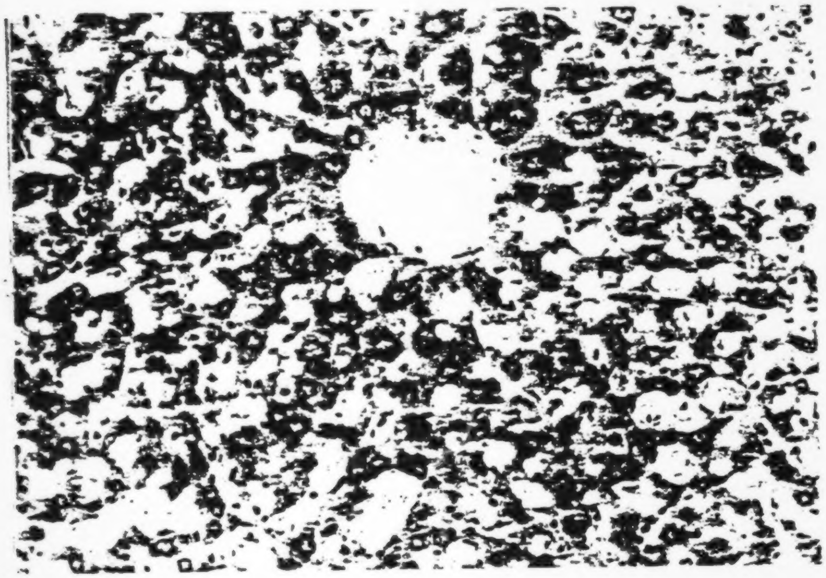

Figure 6: Liver amyloidosis after 104 treatment of 2 μg TPA.

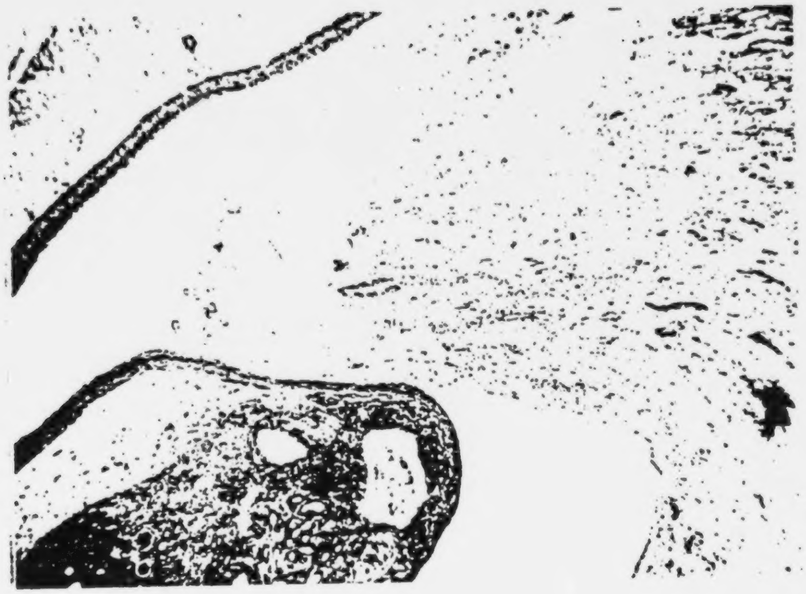

Figure 7: Papillary necrosis seen in the kidney with chronic pyelonephritis after 104 TPA applications.

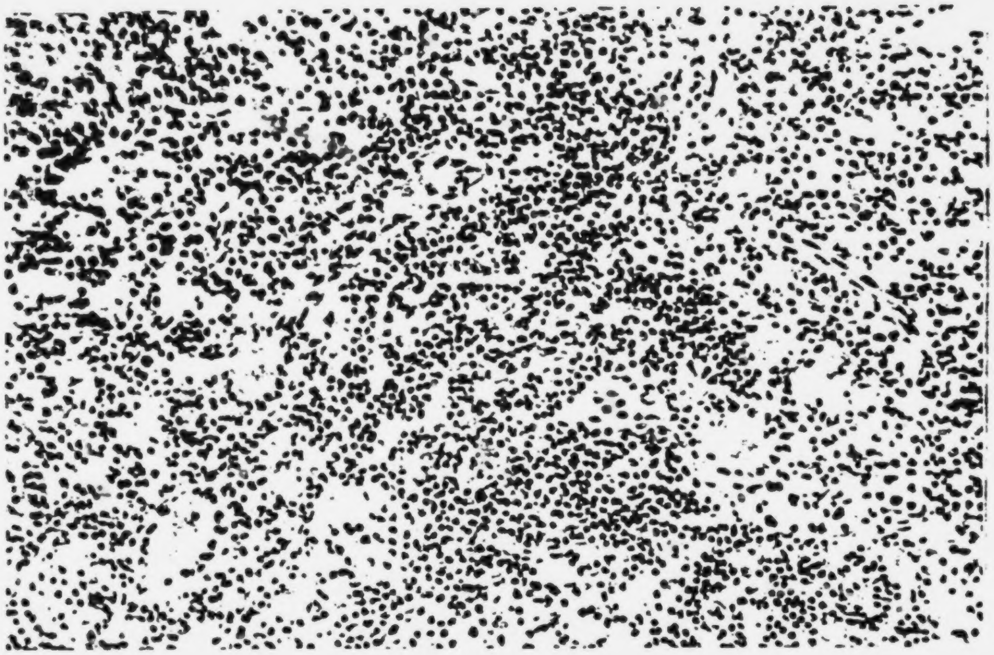

Figure 8: Reactive lymphoid tissue hyperplasia with effacement of the normal lymph node architecture. Note numerous blood vessels. After 104 applications of TPA.

GROSS AND MICROSCOPIC PATHOLOGY IN THE SENCAR MOUSE SKIN AND LUNG:

Initiation and Promotion Studies

by

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Key Words: SENCAR mouse, initiation and promotion, skin pathology, lung pathology, gross pathology, microscopic pathology.

ABSTRACT

During the past three years, the skin and lung tissues from SENCAR mice utilized as part of the Environmental Protection Agency's Carcinogenesis Testing Matrix were examined. This included SENCAR mice used in three different short-term bioassay protocols in which the Skin Papilloma Assay was used to identify initiators, promotors and complete carcinogens. Also included were the pathology findings from SENCAR mice used in the combined bioassay in which both the skin assay and the Lung Adenoma Assay were conducted simultaneously. The gross and microscopic features of treatment-associated and spontaneous lesions of the skin and lung of the SENCAR mouse used in these studies are defined and the lesions most commonly observed are described.

There was generally a poor correlation between gross observations and microscopic findings in both the skin and lung tissues. Although there are several definite criteria on which gross interpretations of the various skin and lung lesions can be made, it was found that with the exception of pedunculated squamous cell papillomas and the classic squamous cell carcinomas, the various lesion types had a wide variety of clinical presentations that severely compromised the accuracy of gross diagnosis. Further, in the case of benign skin neoplasms, it was found that malignant transformation of these tumors most often occurred at the base of the lesion and was initially hidden from gross observation. As a result, approximately 50% of the neoplasms interpreted clincally as benign tumors (papillomas and keratoacanthomas) were actually malignant neoplasms. Moreover, many lesions determined to be non-tumorous grossly were in fact neoplastic microscopically.

The SENCAR mouse was found to be more responsive in the Lung Adenoma Assay than other strains examined with exception of the Strain A. Although accurate interpretation of the lung lesions in the SENCAR was compromised by non-neoplastic treatment-associated and/or spontaneous lesions, the feasibility of using the SENCAR skin and lung as target tissues in two stage combined carcinogenesis studies merits further consideration.

INTRODUCTION

The concept of two stage chemical carcinogenesis was first identified by Berenblum (1) and Rous (2) in 1941. Much of our understanding of the mechanisms of this concept has been gained from the mouse skin papilloma assay (3,4,5,6). Although a variety of strains of mice have been utilized in the assay, the SENCAR mouse, a stock bred specifically for its sensitivity to two-stage skin carcinogenesis, has been most frequently used in these studies. Slaga has shown the SENCAR to be the most sensitive mouse skin carcinogenesis system for the identification of initiatiors, promotors, and more recently, complete carcinogens (7). This sensitivity has been confirmed by other investigators (8,9,10). Based on these findings, the Environmental Protection Agency, Health Effects Research Laboratory, included the SENCAR Skin Papilloma Assay in the bank of short-term bioassays that make up the EPA's Carcinogenesis Testing Matrix, a program of Tier 2 Bioassays designed to confirm the carcinogenic properties of environmental chemicals and mixtures (11). Robinson and others, in an attempt to consolidate several of the assays and improve sensitivity(12), recently reported the use of the SENCAR in a combined bioassay in which the Skin Papilloma Assay was conducted simultaneously with a modified version of the Lung Adenoma Assay, a test traditionally conducted in Strain 'A' mice (13,14).

Although the responsiveness of the SENCAR Skin Papilloma Assay has been reviewed and is well documented for a wide variety of chemicals (4,8,9,10,11,15), and the sensitivity of the lung has been reported in the SENCAR for several chemicals (12), little information has been published on the pathology of the lesions induced in both the skin and lung of SENCAR mice in these assays. Moreover, in the majority of the published reports of skin carcinogenesis in the SENCAR, the pathology has consisted of microscopic confirmation of a percentage of skin lesions or was limited to those identified clinically as carcinomas. There is a paucity of information concerning the diagnostic criteria on which gross observations were based, and microscopically, classification of the skin neoplasms in these reports has been limited to "papilloma" and "carcinoma". In the lung, there are no reports to date concerning the induced or spontaneously-occurring lesions of the SENCAR.

During the past 30 months, we have evaluated by both gross and microscopic examination, the skin and lung tissues from more than 12,000 female SENCAR mice as part of the EPA's Tier 2 Matrix studies for two-stage carcinogenesis. It is the purpose of this paper to provide diagnostic criteria and morphologic descriptions of the gross and microscopic treatment-induced lesions in the SENCAR skin and lung. In so doing, we identify specific shortcomings that occur when results are based exclusively on gross observations without microscopic confirmation.

MATERIALS AND METHODS

Experimental Design

The pathology findings described are representative of treatment-induced lesions observed in female SENCAR mice used in EPA's two-stage Carcinogenesis Testing Matrix project. The four basic types of protocols used in the SENCAR Matrix studies have been previously described in detail (12,15,16,17) and included the following:

Protocol 1: Initiation/promotion (tumor initiators) Skin Papilloma Assay
Protocol 2: Initiation/promotion (promoting agents) Skin Papilloma Assay
Protocol 3: Complete Carcinogenicity Skin Papilloma Assay
Protocol 4: Combined Bioassay; Skin Papilloma and Lung Adenoma Assays.

The first protocol, designed to test the initiating potential of a substance, consisted of the exposure of female SENCAR mice to either single or multiple doses of the chemical or environmental mixture topically or subcutaneously to the shaved dorsum of the mouse. Two weeks following initiation, the promoting agent, 12-0-Tetradecanoylphorbol-13-acetate (TPA) was administered (1 µg/application in 0.2 ml acetone) 3 times a week for 20 weeks. Protocol 2 was the same with the exception that a known initiator, urethrane, was followed by 20 weeks of application with the suspect promotor. For Protocol 3, the test substance was administered 3 times per week to the dorsum of animals, either topically or subcutaneously for a period of 1 to 20 weeks to test for complete carcinogenicity properties. For the Combined Bioassay (Protocol 4), two days after a single dose of a known carcinogen was administered by intraperitoneal injection, 1.0 µg of TPA was applied three times a week for 20 weeks.

The appearance and regression of skin tumors were charted weekly using a grid system with letter and number coordinates to permit specific lesions to be identified by size and topography. For Protocols 1, 2, and 3, the animals were generally sacrificed 12 months following initiation or at approximately 14 months of age. The animals were 36 weeks of age at sacrifice for the Combined Bioassay studies.

Clinical Observations

Following quarantine of the mice, the test animals were identified, weighed, shaved, and placed into treatment groups. The mice were shaved weekly thereafter to facilitate the administering of test compounds and lesion observation. After the initial weighing, the animals were weighed each week for 6 weeks and monthly for the duration of the study.

During the course of the study, 2 daily observations were made of the animals, checking the food and water, and monitoring morbidity. In addition, a weekly observation was performed on all test animals during which all grossly observed lesions were charted on a grid and the lesion type, location and size indexed. Based on clinical observations and standardized morphologic criteria, the lesions were placed by type into one of four categories: (1) papilloma, (2) carcinoma, (3) sore and (4) subcutaneous lesion. During the weekly observations, all lesions identified as tumors were recorded and included in the daily count. When tumors were observed for three consecutive weeks, the tumor counts were included in the cumulative counts. All individual tumors were charted weekly until they regressed or coalesced, or until the animal either died or was sacrificed.

Test Animals

Female SENCAR mice, from 5-8 weeks of age, were obtained from Harlan Sprague-Dawley, Inc., Indianapolis, Indiana. The animals were quarantined at the EPA facility for a maximum of 2 weeks. Mice were placed on study when the majority of mice were in the resting stage of the hair growth cycle. Depending on protocol, the animals were divided into groups of 20 to 40 animals and housed 10/cage. Water and Purina Lab chow were provided *ad libitum.*

Necropsy

A complete necropsy was performed on all spontaneous deaths, morbid sacrifices, and for the scheduled terminal sacrifice. Gross observations of all external and internal lesions were recorded on specially designed necropsy forms that enabled specific documentation of the individual skin lesions as to number, size, and location on the carcass. At least one photograph was taken of each animal to demonstrate each skin lesion and other representative internal gross lesions. Necropsy included examination and fixation of 41 tissues which, along with the carcass, were placed in 10% neutral buffered formalin. In all studies, the lungs were removed intact, examined for lesions, and perfused to approximate tidal volume afterwhich the trachea was ligated. The individual skin lesions were prosected and placed into cassettes to maintain identity. Additionally, a piece of "normal" dorsum skin was also prosected for each mouse. All skin tissues were stretched and mounted on filter paper prior to fixation.

Histopathology

Microscopic evaluation of tissues for all protocols was limited to the dorsal treatment area, all grossly recognized skin lesions up to six per animal, and all other lesions observed as being abnormal either at necropsy or during tissue trimming. In those animals in which multiple skin lesions were present, each lesion was trimmed and processed in a separate cassette in order to maintain identity of the lesion for subsequent correlation with both clinical observations and necropsy findings. In the Combined Bioassay, a Shimkin Adenoma Assay was conducted (13). The lobes of the lung were separated, and lesions consistent with primary lung tumors were counted, tabulated and each lesion was recorded topographically on lung count forms. This task was performed by two technicians working independently of each other. Signficant discrepanies were further examined by either the pathologist or a third technician. Unresolved discrepancies, all gross lesions of questionable diagnosis, and at least 10% of the lung tumors were examined microscopically for lesion confirmation and/or diagnosis.

The identity of the each of the skin lesions was maintained throughout the process to enable subsequent correlation of the microscopic findings with the gross observation at necropsy and the clinical findings recorded during the in-life portion of the study. These data were subsequently analyzed as to the accuracy of diagnoses of the skin lesions based on gross interpretations. All microscopic pathology findings were entered into a computerized pathology data system that generated the individual animal and summary incidence tables.

RESULTS AND DISCUSSION

Gross and Microscopic Lesions of the Skin and Subcutis

Samples of skin tissues removed from the dorsal treatment area and all skin lesions from more than 12,000 SENCAR mice as part of EPA's Matrix studies were examined grossly at necropsy and during tissue trimming, and then evaluated by light microscopy. A wide variety of inflammatory, hyperplastic and neoplastic lesions were observed in the animals. A list of these lesions, separated into general morphologic classifications, is shown in Table 1. The vast majority of these lesions were likely treatment-induced with the exception of focal inflammatory and hyperplastic lesions confined to the superficial aspects of the skin and several neoplasms of the subcutis that were probably naturally occurring.

Non-Neoplastic Lesions of the Skin and Subcutis

The most frequently occuring lesion involving the skin/subcutis in treatment animals was inflammation and hyperplasia of the epidermis and dermis. These changes varied from small focal areas of acute inflammation to extensive erosive and/or ulcerative areas accompanied by varying amounts of epithelial hyperplasia consisting of acanthosis, hyperkeratosis, and parakeratosis (Figure 1). The epidermal rete pegs, consisting of well organized finger-like projections of hyperplastic epithelial cells, extended into the dermis and occasionally the subcutis. Generally, the hyperplastic response was associated with varying amounts of chronic-active inflammation with usually abundant amounts of keratinaceous debris on the epidermal surface. On gross examination, these lesions were clinically recorded as "sores", and were characterized by loss of hair over what was usually a dry, crusty, slightly raised lesion that may or may not have been ulcerated. The margin of the lesion showed a smooth transition as it blended into the adjacent normal skin.

Focal areas of inflammation and hyperplasia were observed in non-treated mice but never to the extent as shown in Figure 1. The small, focal lesions often represented inflammatory response to bite wounds or other local irritation. Other large areas of uniform epithelial hyperplasia not accompanied by either ulceration or inflammation were also observed. These were likely naturally occurring, however, the development of hyperplasia in response to continued shaving, merits consideration. In this paper we provide evidence for the development of neoplasms from hyperplastic lesions, a progression that has been previously documented in mouse skin carcinogenesis studies (18,19,20). Since we have diagnosed squamous cell papillomas in non-treated mice, it is assumed that at least some of the hyperplastic lesions also occurred spontaneously in the SENCAR mouse.

Another non-neoplastic lesion of the skin that was commonly observed in treated animals and in a few of the non-treated mice, was the presence of epidermal inclusion cysts. The incidence of these cysts increased with the severity of skin uleration and the amount of active inflammation. The skin generally remained intact over these lesions and they clinically presented as subcutaneous raised areas or nodules. In those cases where the skin surface was interrupted, the lesion was occasionally misinterpreted as a skin neoplasm. In several instances, the usually thin layer of epithelial cells lining the cyst appeared to undergo malignant transformation, and a squamous cell carcinoma was observed within the cyst. If the skin surface remained intact, the latter lesion was not detected clinically.

A high percentage of the treated mice had aggregates of cellular infiltrates in the subcutis consisting of either plasma cells or loosely arranged aggregates of mature and immature cells of the granulocyte series. The latter lesion, diagnosed as myeloid granulocytic hyperplasia, was a proliferative change that is often systemic and commonly seen in mouse skin paint studies. Historically, this lesion has often been misinterpreted as granulocytic leukemia. The differential features of myeloid hyperplasia versus granulocytic leukemia has been discussed recently by Long (21).

The final non-neoplastic lesion observed in the skin was deposition of amyloid in the dermis immediately adjacent to the basement membrane of the epidermis. This occurred in association with varying amounts of epidermal hyperplasia and usually was accompanied by mild, chronic inflammation of the dermis and subcutis consisting of lymphocytes, macrophages and varying numbers of plasma cells. The presence of amyloid in the skin is not an unexpected finding especially since Kovatch (22) has reported amyloid deposition in a wide range of tissues in both treated and un-treated SENCAR mice.

Lesions of the adnexal structures of the skin were lacking with the exception of focal inflammation of hair follicles. Although small foci of sebaceous cell differentiation were present in several of the skin tumors, hyperplastic lesions of the sebacecous glands were not observed.

Neoplastic Lesions of the Skin and Subcutis

The most frequently occurring benign neoplasm of the skin observed in the SENCAR Matrix studies was the squamous cell papilloma. This tumor had a variety of clinical presentations and varied in size from 0.1cm to greater than 2.0 cm in diameter. The papillomas projected above the surface of the skin either as keratinized pointed protuberances or as lobulated, cauliflower-like growths. The tumors were red to dark tan and had dry, rough and finely fissured surfaces (Figure 2). Papillomas have been classified into two types by the morphologic features of their attachment to the skin; either as pedunculated having a thin stalk, or sessile with a broad base, with their height being less than the diameter of the attachment. Gross examination and palpation of the pedunculated lesion demonstrated the lesion as a knob-like growth attached by a stalk of variable thickness with the base of the stalk attachment blending evenly with the skin surface. The broad based papillomas were more difficult to palpate, but important diagnostic information was gained by carefully examining the base of the lesion for any irregular growths, acute inflammation, hyperemia, swelling and/or adherence to the underlying tissue, all of which are indications of malignancy.

Microscopically, the two types of papillomas consist of similar components but were easily differentiated. The pedunculated papillomas had an outer layer of thickened, relatively regular but folded hyperplastic epidermis capped by varying amounts of keratinized cells and keratinaceous debris. This layer of from 10-20 epithelial cells thick, rested on an inner layer of connective tissue stroma. The dermis within the stalk was often more fibrous and dense than normal, and it generally contained minimal to occasionally severe inflammatory cell infiltrates. The broad based papilloma (Figure 3) had similar components but the lesion lacked a stalk and was less protuberant, having a height less than the diameter of the base of the neoplasm. In both types of papillomas, the cells of the stratum granulosum of the epithelium were often arranged in regular masses, with the development of ridges which were often severely elongated and extended deeply into the dermis. The base of the rete ridges was well organized and the cells were clearly demarcated from the adjacent dermis without interruption of the basement membrane (Figure 4). The neoplasms contained few mitotic figures and the cells were of similar size, shape, and staining qualities. Sectioning artifacts must be considered when examining the base of these lesions, the site where malignant changes most frequently occur. Sometimes nests of cells appeared to be separated from the rete pegs suggesting metastases, but close examination of the cell size, shape and staining properties indicated that the cell clusters did not represent early metastases but occurred rather as a function of cut.

The second type of benign neoplasm of epithelial cell origin that was commonly observed in the SENCAR mice was a keratoacanthoma. These neoplasms, referred to as inverted papillomas, developed in the dermis, arising from the epithelial cells associated with hair follicles. The keratoacanthomas present in the Matrix studies presented a wide variety of gross appearances. Most frequently, these neoplasms had a crater-like appearance, filled with keratin and surrounded by an irregular lip of abruptly reflexed and compressed epidermis (Figure 5). Keratoacanthomas were generally irregularly round, and when situated superficially on the skin, the lesion had a bud-like appearance, pushing completely above the surface and resting on an appendage of stroma in a manner similar to pedunculated papillomas. As reported by Bogovski, the keratoacanthoma is not always easily distinquishable from the papilloma. It is speculated that in these cases the glabrous epidermis and the epithelium associated with the hair follicle simultaneously respond to the carcinogenic action of the same agent(19).

Ghadially (23,24) identified three types of keratoacanthomas in the mouse. Type 1 neoplasms arose from the upper part of the hair follicle, and types 2 and 3 developed from the lower part. Although we have not routinely attempted to characterize keratoacanthomas as to type, it is our experience that approximately half of these neoplasms in the SENCAR were consistent with Ghadially's type 1 tumor, in that they were superficially located and often pedunculated. The remainder of the keratoacanthomas were embedded in the skin as shown in Figure 5. These neoplasms, consistent with the type 3 tumors described by Ghadially, were clinically more difficult to diagnose and were often misinterpreted as ulcers and in some cases, small squamous cell carcinomas.

Descriptions such as "cup- or berry-shaped" commonly used to describe keratoacanthomas were best appreciated when the lesions were examined microscopically (Figure 6). The sides of the tumor were convex and comprised of sheets of compressed epithelial cells. Generally, the central core of the lesion was filled with laminated keratin interrupted only by an occasional frond of epithelial cells. Unlike papillomas, the amount of keratin and keratinaceous debris was usually the predominant component in these lesions. Both trichohyaline and keratohyaline granules were seen in the epithelium. The base of the lesion was situated either in the dermis or subcutis, often resulting in marked displacement and compression of the stroma. This differs from the squamous cell papilloma in that the dermis immediately beneath the papilloma was usually thicker and more dense. In both papillomas and the keratoacanthomas, malignant transformation was observed to occur most often at the base of the neoplasms. Early transformation was characterized by penetration of the compressed dermis and invasion of the muscle layer by nests of irregular cells, distinquished by showing variation in size, shape, and staining qualities. These changes, since they frequently occurred at the base of the tumor and were hidden from gross observation, were seldom diagnosed as malignant lesions except in those cases where significant new tumor growth had pushed to the surface of the skin causing an elevation of the peripheral portions of the tumors.

A review of the clinical and pathology data generated by several of the EPA's Matrix studies indicated that there was a direct correlation between the severity of ulceration during the initial stages of promotion and the incidence of keratoacanthomas. Although the dose of TPA administered in the various Matrix studies did not change, some inconsistency was observed in the response of the skin to promotion. In shipments of mice in which there was early and persistent ulceration in response to promotion, the incidence of keratoacanthomas increased significantly and approximated the number of squmous cell papillomas that developed. In these cases, the malignant transformation of keratoacanthomas was also increased.

The neoplasm routinely having the most varied gross appearance, was the squamous cell carcinoma. The clinical presentation of a classic carcinoma is shown in Figure 7. These neoplasms arose either from pre-existing papillomas or keratoacanthomas, or developed spontaneously within the epidermis, initially as carcinoma in situ, or developed in association with ulcers. If the carcinomas are examined microscopically in the early stages of tumor development, the origin of the lesion can be determined. The most consistent features observed in the carcinomas in our studies, were severe ulceration, crater formation and rapid growth. The latter feature was typically demonstrated by a rapid and striking elevation of the margins of the tumor with the borders becoming indurated and poorly defined.

Based on microscopic features of the neoplasms, three types of squamous cell carcinomas have been previously identified: (a) keratinizing, (b) non-keratinizing, and (c) anaplastic (19). In the EPA Matrix studies, the majority of the carcinomas that developed were keratinizing types. However, it is our experience that the factors that affected the development of keratoacanthomas, similarly influenced the type of carcinoma that developed. In those animals in which there was initially severe ulceration that persisted throughout the promotion phase, the carcinomas that developed were invariably less keratinized and more anaplastic. In these cases, the carcinomas appeared to develop more frequently either from ulcers or within areas of epidermal hyperplasia. The malignant neoplasms appeared to develop spontaneously in association with these lesions without evidence of any benign tumor being present prior to the carcinoma.

Microscopically, squamous cell carcinomas [illegible] significantly in appearance and possessed a wide variety of squamous elements and other, often heterogeneous cellular structures. The appearance of these neoplasms was influenced greatly by the origin of the malignant lesion. Typically, carcinomas developing from papillomas, keratoacanthomas, or epidermal hyperplasia were keratinized type carcinomas, comprised of differentiated and undifferentiated squamous cells arranged in cords or whorls with keratinized or partially keratinized centers (Figure 8). The cords or finger-like trabeculae extended into the underlying dermis, subcutis, and often into the subcutaneous muscle layers and vessels. The cells of these tumors were irregular in shape and size, the nuclei generally round or oval and of varying dimensions. The cells typically stained with varying intensity with those cells at the margins of the neoplasms often being atypical, hypertrophied, and stained pink to brightly eosinophilic. As the carcinomas became less keratinized and more anaplastic, mitotic activity was increased, keratinization of single cells became more frequent, and intercellular bridges, commonly observed in well-differentiated carcinomas, were more difficult to find. Figure 9 demonstrates an atypical squamous cell carcinoma. Poorly developed keratinized squamous elements and aggregates of atypical epithelial cells were observed but the predominant cellular structures were the irregular whorls of poorly differentiated, elongated cells. These structures appeared to be forming rudimentary hair follicles with occasionally, one to several cells in the central core undergoing keratinization.

Associated with the vast majority of carcinomas, regardless of type, was a moderate to severe chronic-active inflammatory process consisting of a mixed population of inflammatory cells and varying amounts of fibrosis. In some cases, there was a striking desmoplastic response, with the reactive connective tissue elements being the predominant structure in the neoplasm. Occasionally, the connective tissue elements of the neoplasms were highly irregular, poorly organized and without orientation. Figure 10 demonstrates such a neoplasm in which the sarcomatous appearing stromal element occupies the majority of the lesion. In several cases, both the epithelial and the connective tissue elements appeared to have undergone malignant transformation, and a diagnosis of carcinosarcoma was rendered. The pathogenesis of a neoplasm of this type is not understood. This may represent two separate and distinct neoplasms that arise simultaneously or may be represent a fibroepithelioma as described by Pinkus (25).

As was the case for several other types of tumors previously discussed, the carcinosarcomas appeared to develop only in those animals that had persistent ulceration in response to either the promotor or initator. The presence of ulceration may have allowed for direct contact of the initiator or promotor with the underlying dermis. Clincally, most of these tumors were diagnosed as "sores" and there was no evidence of a tissue change other than ulceration (Figure 11). The prominant fleshy rim of tumor growth normally associated squamous cell carcinomas was missing.

Interestingly, these lesions were observed only in SENCAR mice that were terminally sacrificed (13 months of age) and not in any animals that died early in the study. This is in agreement with findings in whole-life mouse skin paint studies conducted in other laboratories (26), studies in which, with time, fewer carcinomas were observed, and sarcomas were the predominate tumor type.

Only one basal cell tumor was observed in the Matrix SENCAR mice. This is in agreement with other investigators who report basal cell tumors are rare in mice (19,20). The one basal cell tumor noted in our studies was a malignant lesion, comprised of irregularly round, basophilic cells arranged in dense packets. Within several of the cell clusters, there was primitive sebaceous differentiation.

The remainder of the neoplasms involving the skin were primarily confined to the dermis and subcutis and with few exceptions, were diagnosed clinically as subcutaneous nodules. The vast majority of these lesions were mammary gland neoplasms including both benign and malignant neoplasms. Mammary gland neoplasms in mice have been classified as to predominant cell type (27). The type B mammary gland adenocarcinoma was the most frequent type observed, and mixed adenocarcinomas and adenoacanthomas were also commonly observed types of mammary gland neoplasms.

Among the other neoplasms observed in the subcutis were fibrosarcomas and rhabdomyosarcomas. With the exception of the carcinosarcomas described previously, the various sarcomas of the subcutis appeared to develop totally independent of the superficial aspects of the skin and often only minimal epidermal changes accompanied the neoplasms. The incidence of the subcutaneous sarcomas was signficantly increased in studies in which treatment was administered either by subcutaneous or interperitoneal injection versus the topical route of application. Only one plasma cell tumor was observed in the Matrix mice. Grossly, this neoplasm resulted in irregular elevation of a substantial portion of the dorsum skin. Although the skin generally remained intact over the neoplasm, there was complete loss of hair and severe erythema. Microscopically, loosely arranged sheets of plasma cells infiltrated the dermis and subcutis. This was accompanied by a mild to moderate hyperplasia of the epidermis that consisted primarily of acanthosis. The epidermis was interrupted by multiple erosions and several small focal ulcerations.

Gross and Microscopic Lesions of the Lung

The pathology findings in several of the Matrix studies demonstrated an increased incidence of primary lung tumors in the SENCAR mouse in response to several known carcinogens as compared to vehicle controls. In most cases, this response was dose-related. Based on these findings, Robinson and others (12) subsequently used the SENCAR in combined bioassay studies in which a modified Shimkin Lung Adenoma Assay and the Skin Papilloma Assay were conducted simultaneously. During the past 30 months, the lungs from approximately 3,500 SENCAR mice were examined microscopically. This included the lungs of mice utilized in combined bioassay studies and from mice in other skin initiation-promotion studies in which abnormal lung changes were recognized grossly. The various types of lesions that we observed in the SENCAR lung are shown in Table 2.

Non-Neoplastic Lesions of the Lung

A wide variety of inflammatory lesions were the most frequently observed non-neoplastic changes observed in the lungs of SENCAR Matrix mice. These inflammatory responses varied in both morphologic features and severity and in several studies, the changes were not always treatment and/or dose related. In the latter case, the lesions were non-specific in origin. The most common lesion consisted of randomly distributed peribronchiolar and perivascular lymphocytic cellular infiltrates that generally involved from one to several pulmonary airways and/or vessels. In some cases, there was extension from the collar of lymphoid infiltrates into the adjacent alveolar interstitium for short distances. Frequently, a small population of plasma cells accompanied these lesions. Similar changes have been described in other stocks and strains of mice and were regarded as common, nonspecific and incidental findings.

In several Matrix studies, the pulmonary response consisted of a more severe, chronic-active interstitial inflammation. Lymphocytes, plasma cells and macrophages infiltrated the interstitium and there was often early fibrosis of the alveolar septa. Varying numbers of foamy histiocytes occupied the alveolar spaces and occasionally fewer numbers of neutrophils and lymphocytes were present. Associated with these changes was a minimal to mild hyperplasia of the alveolar lining cells. The morphologic features of these lesions were similar to those described as being caused by infectious agents including Sendai infection (28). However, no definitive agent was identified by serologic examination of sentinel mice. These lesions on gross examination, appeared as poorly defined, irregularly round tan foci, and when subpleural, they were slightly raised above the pleural surface of the lung. These subpleural changes were difficult to distinquish from primary lung tumors on gross examination and during the Shimkin counts.

Another type of inflammatory response observed was more granulomatous in nature and often included the formation of one to several granulomas that had varying degrees of organization. These lesions were occasionally quite large and were almost exclusively subpleural in distribution. The presence of these lesions was restricted to those mice that had severe skin ulcerations and/or ulcerated skin tumors. In these animals, there were also frequently granulomas or chronic inflammatory infiltrates in the kidney and/or liver. These lesions likely represented the response to systemic infections that originated from the ulcerated skin. In the lung, the well-organized subpleural granulomas were misinterpreted routinely as being primary lung tumors in that they were generally tan, well circumscribed and were elevated above the pleural surface of the lung, features consistent with pulmomary adenomas.

Histiocytosis, in the absence of other inflammatory response, was also a frequent observation in the lungs of both treated and non-treated SENCAR mice. This lesion, recognized routinely in other strains and stocks of mice, has been interpreted as an incidental, nonspecific change, often associated with older mice (29). The lesions in the SENCAR mouse, in the absence of other inflammation or neoplasia, consisted of large histiocytes (macrophages) with abundant grey foamy cytoplasm and a single small dark nucleus. In mice with primary lung neoplasms, aggregates of histiocytes were at the margins of tumors, especially in association with alveolar-bronchiolar carcinomas. In these cases, the cytoplasm of the histiocytes was brightly eosinophilic and the nucleus appeared pyknotic. The presence of cells of similar morphology have been reported in association of alveologenic tumors in many strains of mice (29). In the SENCAR mice, brightly eosinophilic histiocytes were also found associated with small focal areas of alveolar lining cell hyperplasia in which the alveolar spaces contained both histiocytes and a homogenous, proteinaceous material that likely represented a secretory product. When these lesions had a subpleural distribution, they were occasionally misinterpred as being primary lung tumors.

Myeloid hyperplasia was observed in the lung of SENCAR mice that had severe, long standing, skin ulcers or ulcerated neoplasms. Mature and immature cells of the granulocytic series were within alveolar capillaries and major pulmonary vessels. This lesions seldom had gross manifestations.

Hyperplasia of either alveolar lining cells and/or the bronchiolar epithelium was a common observation in the Matrix mice. These hyperplastic foci were most frequently observed in association with alveolar ducts, and to a lesser extent, with terminal bronchioles. They were seldom subpleural in distribution. Some investigators have previously classified the pulmonary hyperplasias as to overall morphologic pattern and have included types such as solid, tubular, papillary, alveolar, etc. (30,31). The vast majority of the hyperplastic lesions observed in the SENCAR lung were of the alveolar and/or papillary-mixed types.

Neoplastic Lesions of the Lung

The classification of primary pulmonary tumors in the mouse lung continues to be controversial. Most pathologists involved in mouse carcinogenesis studies have separated the alveolar-bronchiolar tumors into adenomas and carcinomas. However, it is generally accepted that pulmonary adenomas ultimately become malignant (32,33). In the SENCAR Matrix mice, both alveolar-bronchilar adenomas and carcinomas were observed, but the majority of the primary lung tumors were adenomas. This was likely influenced by the age of the mouse and the time on study since all Matrix mice were sacrificed before 14 months of age with some studies terminated before 12 months. Ward (30) and others (30,31,32,33,34,35) have classified the primary lung tumors by type according to the predominate morphologic arrangement. Solid, papillary, tubular, alveolar and mixed types have been identified. In the SENCAR Matrix mice, approximately equal numbers of solid and papillary or papillary-mixed were observed. The solid alveolar-bronchiolar adenomas were most frequently subpleural in distribution and were easily discernable on gross examination as pale, tan well-circumscribed elevated nodules. Microscpically, these lesions consisted of tightly packed, non-ciliated cuboidal cells that rested on scanty amounts of fine reticulin stroma. The borders of these neoplasms compressed the adjacent parenchyma and were often poorly defined and non-encapsulated. The majority of the papillary and/or papillary-mixed type adenomas were associated with small airways. In some cases, it was impossible to discern whether the tumors originated in the bronchiole or if the neoplasm had grown into the airway. In these lesions, the neoplastic cells were columnar, devoid of cilia, and uniform in size and shape. The borders of these lesions were also poorly defined. Neoplastic elements feathered into the adjacent parenchyma in a manner resembling hyperplastic epithelial growth.

The malignant primary lung tumors, alveolar-bronchiolar carcinomas, varied in size from small focal tumors consisting of highly anaplastic cells to large neoplasms that effaced entire lung lobes. In that large tumors, it was not uncommon to see multiple morphologic patterns in one lesion. The majority of the carcinomas observed in the Matrix mice were mixed types and frequently airway-associated. Our observations indicated that the papillary or papillary-mixed type neoplasms were more likely to become malignant lesions than the subpleural solid tumors. In some of the smaller papillary or mixed tumors, small foci of anaplastic cells, usually more basophilic than the adjacent tumor cells, were observed as islands within a well-differentiated adenoma. These islands likely represented malignant transformation of the lung tumor, a finding previously described by Mori (36). Mild to occasionally severe desmoplastic response was associated with the larger carcinomas. Although vascular invasion by malignant cells was observed, no metastases to other organs was observed.

The cell of origin for primary lung tumors in the mouse has also been much debated. Until recently, most investigators reported pulmonary tumors in the mice as arising from alveolar lining cells, more specifically, the type II lining cell (29,32.33) However, the finding that some of the neoplasms arose in or adjacent to airways suggested that bronchiolar epithelial cells may be the origin for some pulmonary tumors. Kaufman et al. (34, 35) first proposed the Clara cell as the cell of origin for tubular and/or papillary lesions that were most frequently airway-associated. Most recently, Ward (30,31) used avidin-biotin immunocytochemical procedures to localize surfactant apoprotein (SAP) and Clara cell antigen (CCA) in both spontaneous and induced lung tumors in the several strains and stocks of mice including the SENCAR. These investigators found that the majority of the pulmonary tumors in the mouse were immunoreactice to SAP, a marker for alveolar type II cells and that the Clara cell antigen could not be detected in any lung tumors, even in the papillary or tubular airway-associated tumors previously reported as being of Clara cell origin (34, 35). It appears that the cell of origin for the solid, subpleural tumors and the papillary or papillary-mixed airway-associated neoplasms described in the SENCAR Matrix mice, remains controversial.

The only other primary lung neoplasm observed in the SENCAR mice was a squamous cell carcinoma. These tumors are extremely rare in mice (32) but have been induced by a number of polycyclic aromatic hydrocarbons (33). The one squamous cell carcinoma observed in the Matrix studies occupied all of one lobe of lung and extended into the adjacent pleura. Microscopically, the neoplasm consisted of sheets of anaplastic cells and there were numerous mitotic figures. Unlike previously reported squamous cell carcinomas (32), the neoplasm in the Matrix study contained only small amounts of poorly developed keratin with only limited formation of keratin pearls. Morphologically, this tumor resembled the foci of pulmonary squamous cell metastases that was present in several mice with skin carcinomas. However, in the mouse with the primary neoplasm, no neoplastic lesions were present in either the skin or in the esophagus and forestomach.

Metastatic or secondary neoplasms involving the lung were observed in several of the Matrix mice. Metastases from mammary gland neoplasms and from malignant skin lesions were most frequently observed. In these cases, the areas of metastases could not be differentiated by gross examination from primary pulmonary tumors.

Correlation of Gross and Microscopic Findings

During our discussion of the different types of lesions involving the skin and lung of the SENCAR Matrix mice, an attempt was made to define the gross and microscopic morphologic features that were most commonly associated with specific lesions. These morphologic characteristics were used routinely in rendering clinical diagnoses during the in-life portion of each study, during the recording of gross observations at necropsy, and during microscopic tissue evaluation. As part of the final pathology report that was generated for each Matrix study, a correlation table was prepared that compared the gross necropsy observations and the microscopic findings. Recently, we expanded the correlation of gross and microscopic data to include not only the gross findings recorded at necropsy, but also the clinical data recorded at the last observation period prior to terminal sacrifice. This observation occurred usually one week and not more than one month prior to sacrifice. Although a definitive statistical analysis of the correlation of the final clinical observation and microscopic data was not included in this report, a review of the clinical and microscopic data indicated that there were obvious shortcomings in the diagnostic accuracy of the gross observations.

It is our experience that even when the same personnel are utilized and their training maintained consistently, and the diagnostic criteria well established, many squamous cell skin neoplasms cannot be diagnosed accurately when only gross criteria are applied. The lack of sensitivity of in-life and/or gross observations is particularly disconcerting when clinical observations are not supported by microscopic findings. Historically, attempts to correlate gross and microscopic diagnoses in skin initiation-promotion studies were either not performed or reported and when provided, the data were generally limited to confirmation of those lesions grossly determined to be carcinomas. It has been our experience that there was a high correlation between the lesions diagnosed grossly as squamous cell carcinomas and the microsopic findings. In these cases, the type of tumor and its maligant properties were generally easily recognizable. In one Matrix study, 18 of the 19 skin lesions diagnosed grossly as squamous cell carcinomas, were microscopically confirmed as malignant neoplasms. Hennings (37) recently reported that in excess of 90% of the tumors diagnosed clinically as carcinomas were microscopically confirmed as squamous cell carcinomas. In most of these reports, the microscopic confirmation was limited to those lesions diagnosed grossly as carcinomas. This may explain the low conversion rates of papillomas to carcinomas that have been reported (38) since many of the lesions may have had subtle malignant changes that were not detected grossly and were never examined microscopically.

Correlation of clinical observations with microscopic findings also indicated that pedunculated squamous cell papillomas were also accurately diagnosed by gross examination, especially if the lesions were palpated carefully. The data from selected studies examined indicated that pedunculated squamous cell papillomas, as observed at the final in-life observation point, were diagnosed with 92% accuracy (44 of 48 papillomas).

The diagnostic accuracy of gross observations was decreased markedly when other types of skin lesions were considered. Particularly troublesome were sessile papillomas, keratoacanthomas, and lesions diagnosed during in-life observation as sores. Almost half of the sessile papillomas (12 of 26) and keratoacanthomas (4 of 10) diagnosed grossly were microscopically confirmed to be squamous cell carcinomas. Six of ten lesions diagnosed grossly as sores were microscopically diagnosed as squamous cell carcinomas or carcinosarcomas. The reduced clinical diagnostic accuracy for lesions of this type can in part be explained by the fact that the early malignant transformation in these lesions most frequently occurred at the base of the lesion and was hidden from gross observation. It was not until the neoplastic cells extended upward and to the margins of the tumor was the malignant change appreciated grossly. It appears that the poor clinical diagnostic accuracy of lesions of this type was not limited to our laboratory. Ward reported that in three studies examined, there were 3-4 times more carcinomas diagnosed histologically than were found clinically and it was recommended that all skin tumors be examined microscopically when malignancy is a critical endpoint (31).

Figure 11 demonstrated a lesion diagnosed clinically as a sore that was actually a carcinosarcoma. If the rim of fleshy malignant tissue that usually was present at the margins of the ulcerated portion of a carcinoma was missing, the lesions were inaccurately diagnosed as a sore. The data indicated that approximately 50% of the sores were actually carcinomas. We did observe that if the ulcerated lesion was dry and crusty, the lesion was more often an ulcer with epithelial hyperplasia rather than neoplasia. Occasionally, a skin lesion was observed that had the gross appearance of a small sessile papilloma (Figure 12). However, microscopically, although superficial papilliferous protuberance was present, the base of the tumor consisted of a highly malignant squamous cell carcinoma that had invaded the subcutaneous muscle and body wall (Figure 13). The anaplastic squamous cell carcinoma shown in Figure 9, was clinically diagnosed as a papilloma. This lesion, shown microscopically in Figure 14, consisted of a superficial protuberance that was consistent with a papilloma, however, the majority of the lesion consisted of a highly anaplastic carcinoma that had invaded all layers of the skin. It is unlikely that this lesion could have been diagnosed accurately without at least gross sectioning. Similarly, in Figure 15, there was malignant conversion of a broad based papilloma. In this case, the change was subtle, and even with careful palpation and gross sectioning, it was impossible to detect the maligant lesion.

In some cases, the changes were so subtle that careful microscopic evaluation was necessary to identify the malignant changes. Figure 16 demonstrates the base of neoplasm in which there are multiple islands of malignant cells that have developed separate from base of the rete ridges, the area in which malignant transformation most frequently occurred. Figure 17 demonstrates even more vividly the frequent microscopic subtleness of malignant transformation. Although there was indication of junctional activity at the basement membrane, the definitive diagnosis of squamous cell carcinoma was made based upon the two small aggregates of malignant cells identified by arrows. Multiple sections of this lesion were prepared and in each, one or several small aggregates of malignant cells were observed.

Similar gross versus microscopic correlation difficulties were also encountered in the lung of SENCAR Matrix mice. In particular, during the conduct of the modified Shimkin procedures as part of the combined bioassay studies, inflammatory lesions were misinterpreted as being primary lung tumors. Subpleural granulomas and focal areas of chronic-active interstitial inflammation were commonly misinterpreted grossly as being primary lung tumors as were metastatic lesions from the skin, mammary gland and other tissues. Accurate gross interpretation of the alveolar-bronchiolar tumors was further compromised by the distribution of the primary lung tumors. Approximately half of the lesions were loosely-arranged papillary or papillary-mixed, airway-associated adenomas located deep in the lung parenchyma. This differs from the Strain A mouse routinely used in the Lung Adenoma Assay in that the vast majority of the Strain A primary lung neoplasms were solid, well-defined, subpleural nodules and were easily recognized grossly (12, 13, 14). In one combined bioassay study in which both SENCAR and Strain A mice were utilized, only 30% of the lesions diagnosed as tumors grossly could be microscopically confirmed as primary lung neoplasms. Routinely, more than 75% of the lung tumors diagnosed grossly in the Strain A mice are microscopically confirmed. Not only does the subpleural location of the majority of the Strain A aid the accuracy of the Shimkin procedures, but also the inflammation present in the SENCAR mouse lung that frequently compromised gross diagnosis was not present in the Strain A. However, Robinson and others (12) have reported that after microscopic examination was performed on the lungs of treated SENCAR mice, the SENCAR was only slightly less sensitive than the Strain A in response to several known carcinogens and was signficantly more responsive than other strains examined.

CONCLUSION

The gross and microscopic morphologic features of the various treatment-induced and spontaneously occurring lesions of the skin and lung of the SENCAR mouse have been reviewed. With few exceptions, there was generally poor correlation between the gross observations and the microscopic findings despite careful clinical observation and palpation. For the most part, gross versus microscopic discrepancies in the interpretation of skin lesions were attributed to the wide range of gross manifestations the various lesions presented and when there was apparent malignant transformation of benign skin neoplasms, it most frequently occurred at the base of the lesion and could not be detected intially by gross observation. In the lung, non-neoplastic treatment-associated and spontaneous background lesions often compromised accurate gross interpretation of primary lung tumors. However, the results of combined bioassay studies indicated that the SENCAR lung was responsive to several known carcinogens and that the SENCAR skin-lung tumor combined bioassay merits further consideration.

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TABLE 1. LESIONS OF THE SKIN AND SUBCUTIS

NON-NEOPLASTIC LESIONS

1. Epithelial Hyperplasia (acanthosis, hyperkeratosis, parakeratosis)
2. Inflammation (acute, subacute, chronic, chronic-active)
3. Erosion
4. Ulceration
5. Epidermal Inclusion Cyst
6. Myeloid Hyperplasia
7. Amyloid

HISTOLOGIC TYPES OF NEOPLASMS

EPIDERMAL NEOPLASMS

1. Squamous Cell Papilloma
   (a) pedunculated
   (b) sessile (broad based)

2. Keratoacanthoma
   (a) type I
   (b) type II
   (c) type III

3. Squamous Cell Carcinoma
   (a) keratinizing
   (b) non-keratinizing
   (c) anaplastic

4. Basal Cell Tumor

OTHER NEOPLASMS OF THE SUBCUTIS

1. Fibroma
2. Fibrosarcoma
3. Rhabdomyosarcoma
4. Plasma Cell Tumor
5. Sarcoma, NOS.
6. Malignant Lymphoma

TABLE 1. CONTINUED.

MAMMARY GLAND NEOPLASMS

1. Adenoma
2. Adenocarcinoma
3. Adenoacanthoma

TABLE 2. LESIONS IN THE LUNG

NON-NEOPLASTIC LESIONS

1. Lymphoid Infiltrates (peribronchiolar and perivascular)
2. Inflammation, interstitial
3. Inflammation, granulomatous
4. Histiocytosis
5. Myeloid hyperplasia
6. Hyperplasia, alveolar lining cell
7. Hyperplasia, bronchiolar lining cell

PRIMARY NEOPLASMS OF THE LUNG

1. Alveolar-Bronchiolar Adenoma (Type II and Clara)
   (a) solid
   (b) papillary
   (c) tubular
   (d) alveolar
   (e) mixed

2. Alveolar-Bronchiolar Carcinoma

3. Squamous Cell Carcinoma

SECONDARY NEOPLASMS OF THE LUNG

1. Mammary Adenocarcinoma
2. Squamous Cell Carcinoma
3. Histiocytic Sarcoma
4. Sarcoma, NOS.

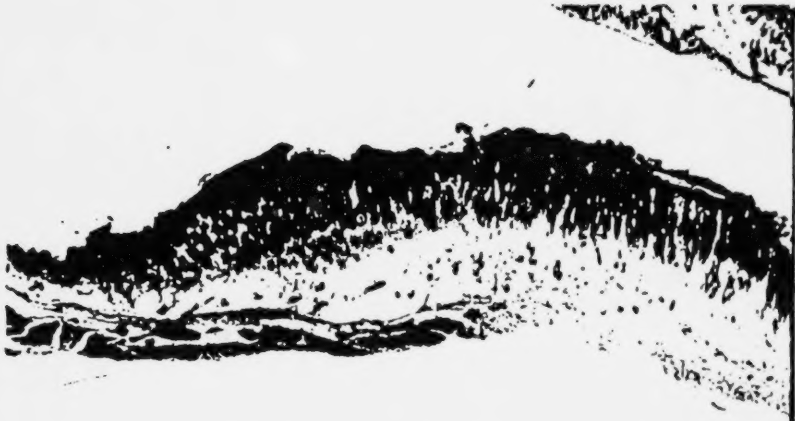

FIGURE 1. Epithelial hyperplasia. Area of severe epithelial hyperplasia consisting of acanthosis and hyperkeratosis with inflammation of the dermis and subcutis. Normal skin is observed at the left margin of the lesion (H & E, x 26).

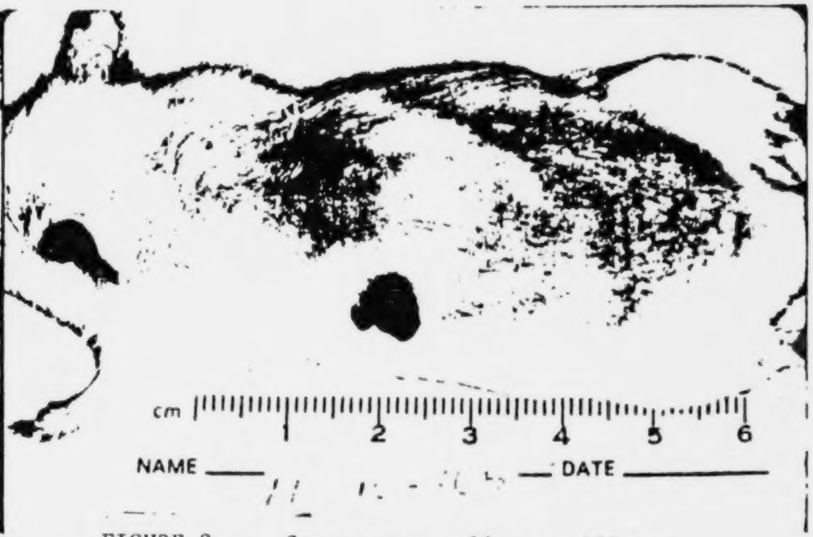

FIGURE 2. Squamous cell papilloma. Typical sessile papilloma in a SENCAR mouse with keratinized, cauliflower-like protuberances.

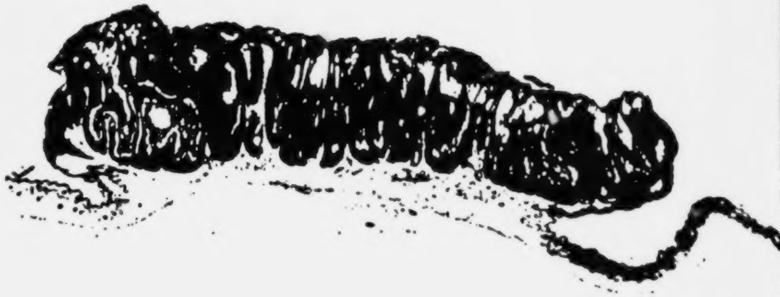

FIGURE 3. Squamous cell papilloma. Typical sessile (broad based) squamous cell papilloma with the diameter of the base of the tumor greater than the height (H & E, x 26).

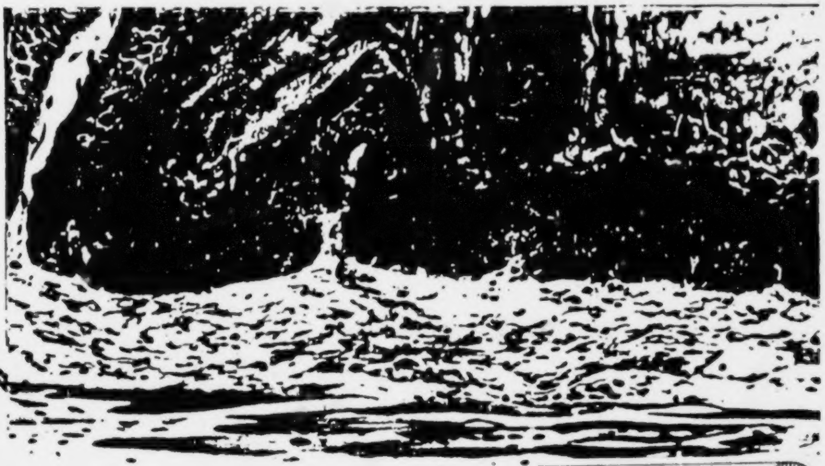

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FIGURE 4. Squamous cell papilloma. Base of the sessile papilloma in Figure 3. The base of the rete ridges are well organized and there is no interruption of the basement membrane (H & E, x 260).

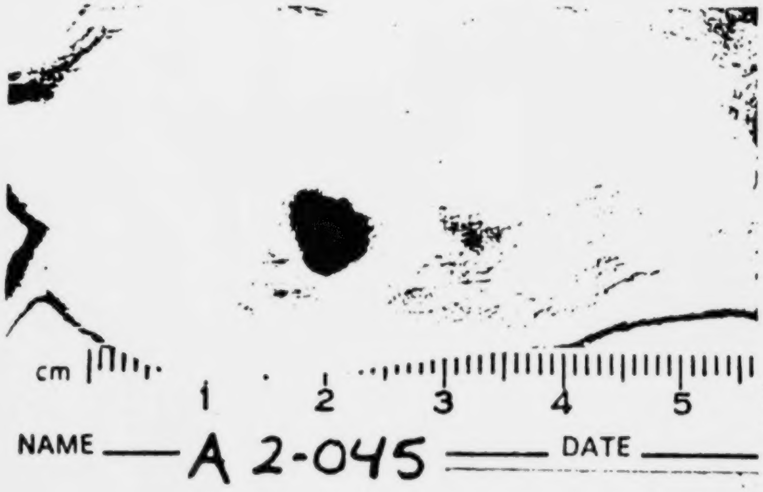

FIGURE 5. Keratoacanthoma. Irregularly round slightly raised neoplasm having a central crater surrounded by an elevated rim of epidermal tissue.

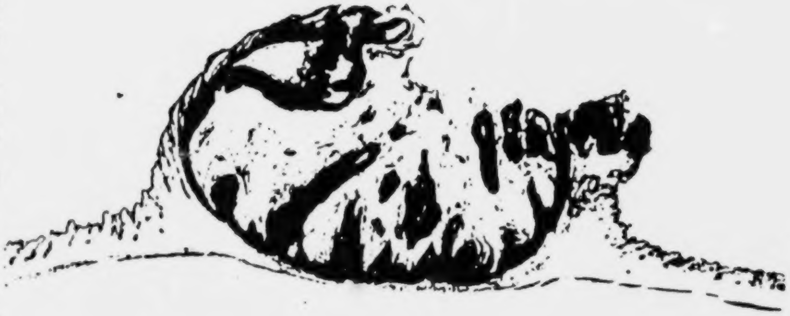

FIGURE 6. Keratoacanthoma. Classic cup-shaped keratacanthoma. There is sharp demarcation between the margins of the tumor and the adjacent subcutaneous tissue. (H & E, x 26).

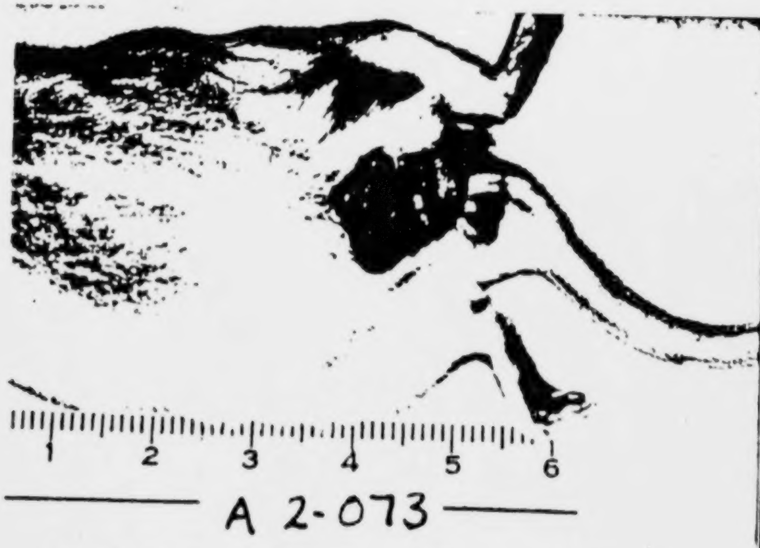

FIGURE 7. Squamous cell carcinoma Carcinoma with crater formation central ulcerations with the margins formed by a rim of elevated malignant epidermal tissue.

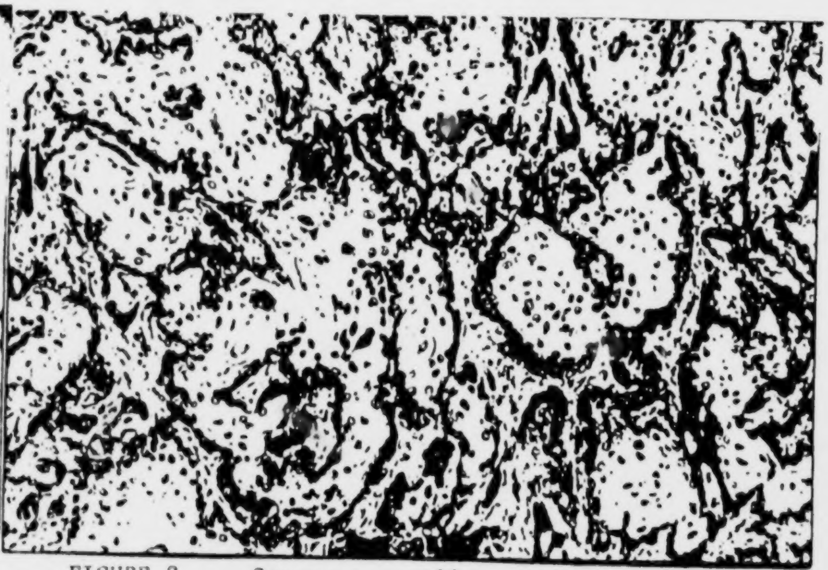

FIGURE 8. Squamous cell carcinoma. Differentiated and undifferented squamous cells are arranged in irregular cords and whorls with keratinized and partrially keratinized centers. (H & E, x 260).

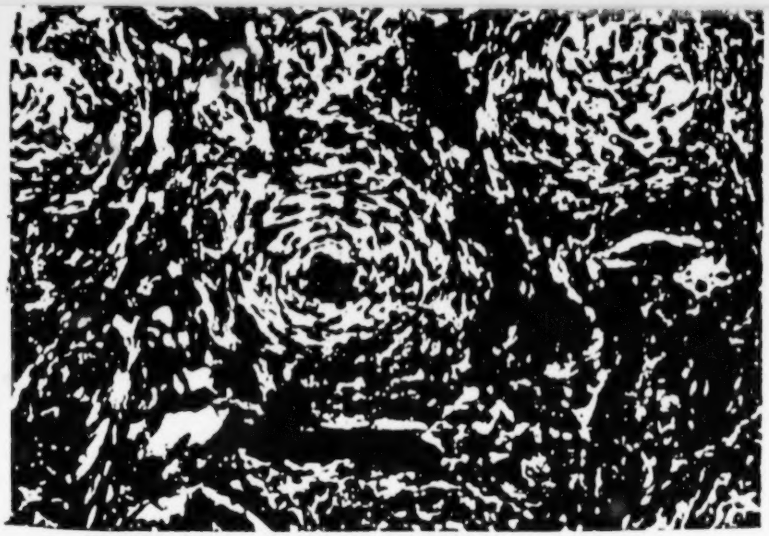

FIGURE 9. Squamous cell carcinoma. Anaplastic squamous cell carcinoma with irregular whorls of poorly differentiated elongated cells. Cells appear to be forming rudimentary hair follicles with keratinization of the core. (H & E, x 260).

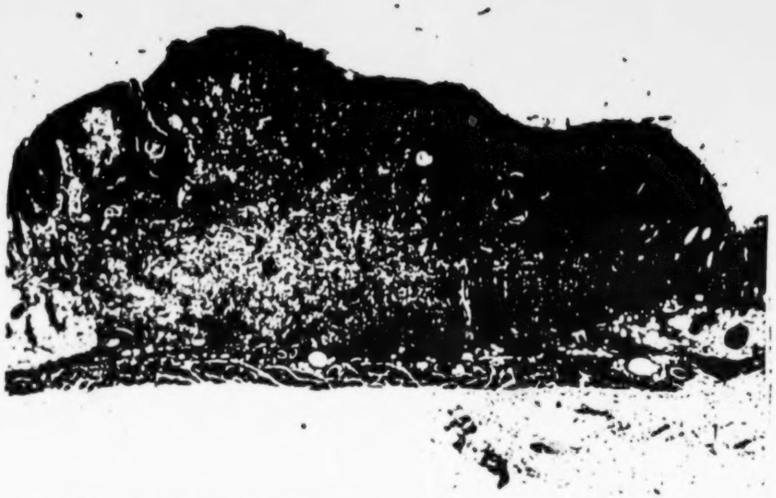

FIGURE 10. Squamous cell carcinoma. Carinoma in which the sacrcomatous appearing stromal element of the neoplasm occupies the majority of the lesion (H & E, x 52).

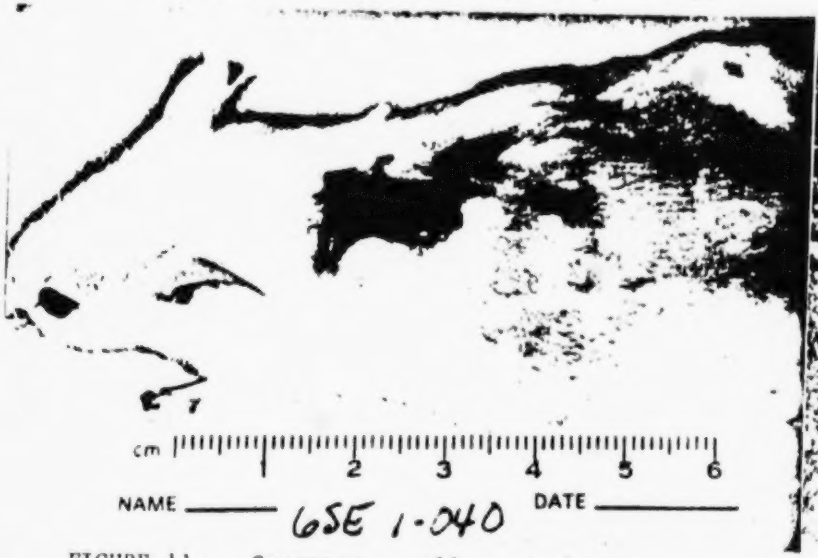

FIGURE 11. Squamous cell carcinoma. Ulceration, typical of a carcinoma is present, but the elevated rim of malignant tissue at the margins of the lesion is absent. Lesion was clincially diagnosed as a sore (ulcer).

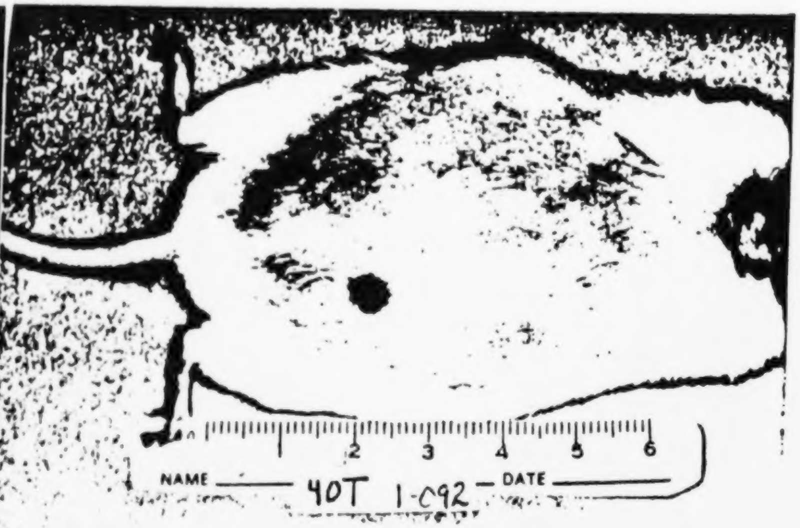

FIGURE 12. Squamous cell carcinoma. The lesion has the clinical presentation of a sessile papilloma. The malignant transformation at the base of the lesion was not observed grossly.

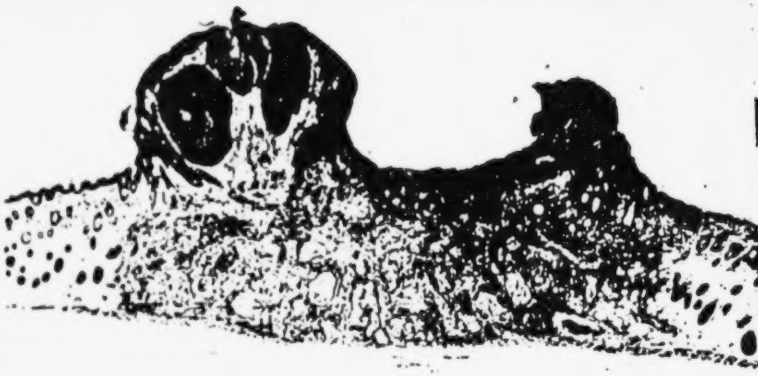

FIGURE 13. Squamous cell carcinoma. Microscopic appearance of the neoplasm in Figure 12. Papillary protuberances border the lesion but there is invasion of the skin and body wall by the malignant neoplasm at the base of the lesion (H & E, x 52).

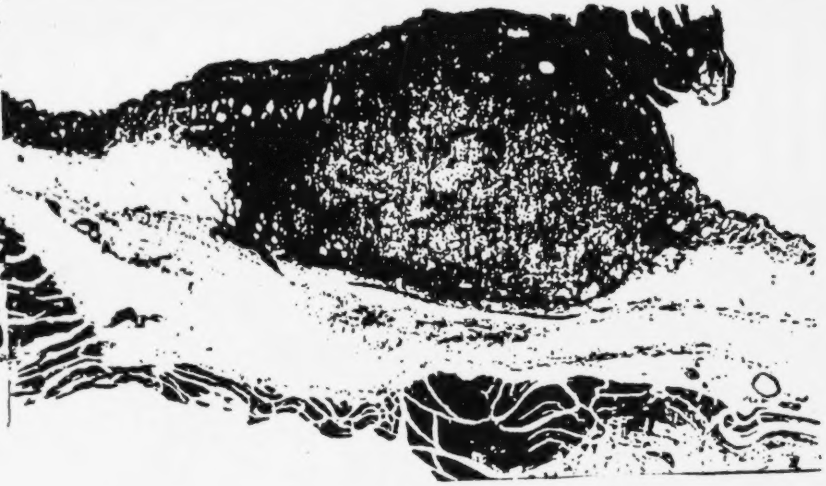

FIGURE 14. Squamous cell carcinoma. Lower magnification of anaplastic carcinoma in Figure 9. The neoplasm had the superficial appearance of a typical, small papilloma. (H & E, x 52).

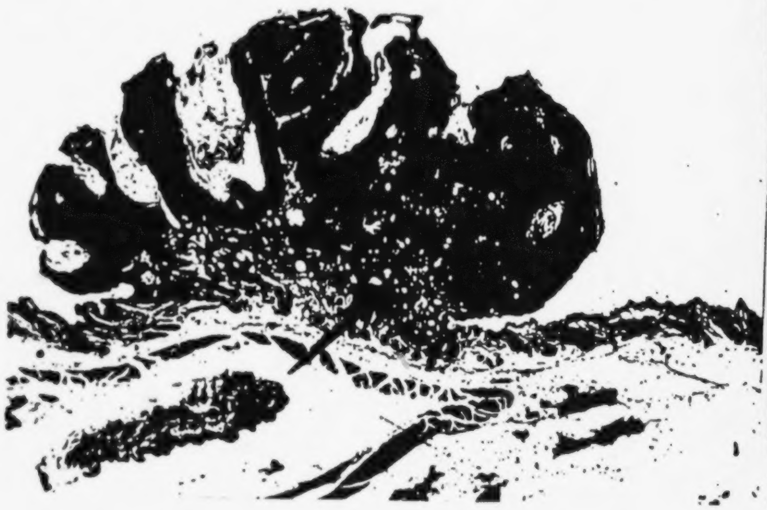

FIGURE 15. Squamous cell carcinoma. Malignant transformation at the base (see arrow) of a sessile papilloma (H & E, x 130).

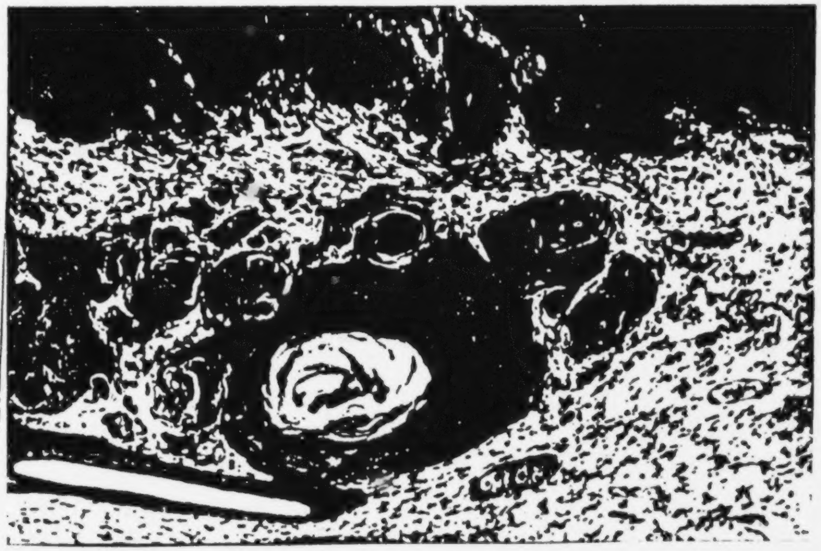

FIGURE 16. Squamous cell carcinoma. Several aggregates of malignant squamous cells at the base of a papilloma (H & E, x 130).

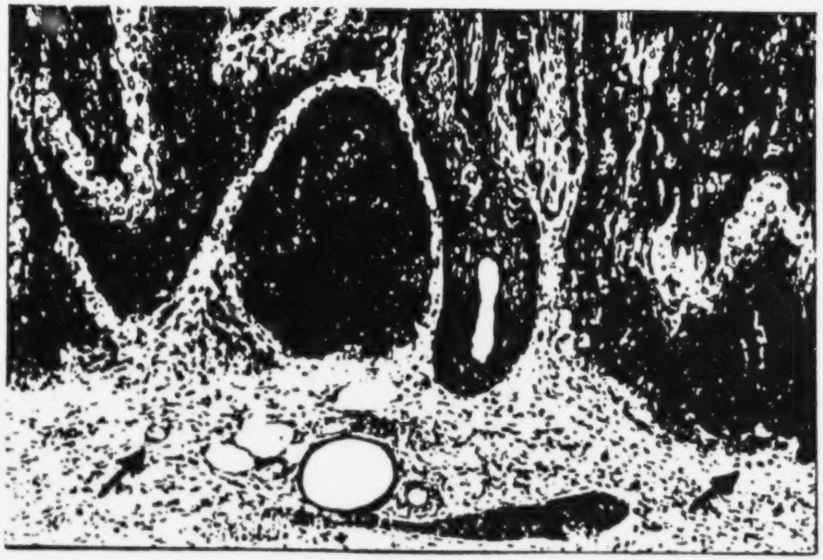

FIGURE 17. Squamous cell carcinoma. Subtle malignant transformation of a lesion at the base rete ridges. Note two small aggregates (arrows) of malignant cells separate from the ridges. (H & E, x 130).

NATURALLY OCCURRING NON-NEOPLASTIC PATHOLOGY IN THE FEMALE SENCAR MOUSE

by

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Key Words: SENCAR Mouse, Pathology

ABSTRACT

Lesions, that were considered naturally occurring; were surveyed in female SENCAR mice ultilized in short term bioassays. These lesions were encountered in protocol designated target organs, and organs and tissues with gross lesions. The Genitourinary system was the most frequent site for lesions where cystic ovaries, cystic endometrial hyperplasia and glomerulonephritis were commonly encountered.

INTRODUCTION

SENCAR mice have been developed for susceptability to skin tumor response utilizing a 2-stage tumor induction regimen (1,2). A paucity of information is available on the naturally occurring lesions in this strain. It is the purpose of this paper to present a survey of lesions encountered in control and treated female mice used in short-term SENCAR Mouse EPA Carcinogenesis Testing Matrix Studies.

MATERIALS AND METHODS

SENCAR mice were obtained from Harlan Sprague Dawley Laboratories, Indianapolis, Indiana. During the experiment mice were grouped 10 per cage and fed Purina laboratory chow and water ad libitum.

Mice on these studies include control and test animals that have an 8-week pre-treatment, and a 30 or 52 week treatment phase. Tissues examined include selected target organs and all organs or tissues with gross lesions encountered in a complete necropsy of each animal. Organs and tissues were fixed in 10% formalin, processed through paraffin, sectioned at 5 or 6 microns and stained with hematoxylin and eosin for microscopic examination.

RESULTS AND DISCUSSION

Digestive System

Liver: The presence of amyloid-like deposits were found in the liver of both test and control mice (Fig. 1). It appeared as slightly eosinophilic amorphous deposits located around sinusoids. Deposits were most intense in peripheral portions of the lobule. Such lesions were usually associated with inflammatory processes elsewhere in the body, such as clitoral gland abscesses and in skin paint studies wherein an extensive inflammatory reaction was associated with the skin lesion. Generally, the amyloidosis was systemic and involved multiple organs as described below. Amyloidosis is not an uncommon lesion in mice and has been reported in a variety of mouse strains (3).

In this age group foci of cellular alteration considered to have preneoplastic significance were uncommon. However, these foci need to be differentiated from foci of vacuolated, usually lipoid laden, cells that involve one or a few hepatic lobules that are found near the cleft of the median lobe (Fig. 2). In my experience the latter lesion is non-age related, is encountered in several species, and does not have any preneoplastic significance.

Inflammatory cell infiltration or hematopoietic cell proliferation commonly occurs in the liver. The inflammatory reactions appear as aggregates of lymphocytes and macrophages distributed randomly throughout the liver. These have not been associated with a specific etiological agent in our studies. Chronic reactions commonly have brown pigment, probably hemosiderin or bile, present in small lobes; the latter are attributed to bile duct obstruction. Hematopoietic cell proliferation presents as variably sized erythrocytic, granulocytic or mixed foci in sinusoids of the liver. These were common in tumor-bearing mice or in mice with inflammatory processes elsewhere in the body. The hematopoiesis was extensive in many instances giving the appearance of leukemia as described by Long (7).

Strangulation of the liver lobes, or portions thereof, were seen both grossly and microscopically. Some of these lesions were misinterpreted as pancreastic masses by inexperienced prosectors. Microscopically such areas of ischemic liver necrosis are frequently surrounded by a prominent connective tissue capsule (Fig. 3).

Other lesions encountered included biliary cysts, fatty change in debilitated animals, focal sinusoidal ectasia, and lymphocytic or plasmacytic infiltrates of portal areas.

Pancreas: Focal acinar cell atrophy was observed. The lesion was characterized by depletion or absence of acinar cells with only pancreatic islets and ductules remaining in some cases. Amyloid-like deposits were occasionally observed histoloically and appeared diffusely in interstitial areas but tended to concentrate near small vessels. Also seen were pancreata with a diffuse white appearance grossly. Zymogen granule depletion was the prominent microscopic feature (Fig.4). Focal islet hyperplasia was the only other alteration found in this survey.

Digestive Tract: Inflammatory reactions were infrequent. Small mucosal erosions and ulcerations were occasionally found (Fig.5). Also hyperplasia of gut associated lymphoid tissue of the small and large intestine was infrequently seen. Pinworms were infrequently identified in the lumen of the large intestine.

An unusual glandular hyperplasia of the duodenal mucosa was observed. Only a few examples were found and they occurred in test animals. The lesion was characterized by a segmental hyperplasia of the glandular elements with ectasia of glands near the base (Fig. 6).

Endocrine System

Adrenal gland: Adrenal gland focal segmental hyperplasia extending from the zona granulosa and hypertrophy of small nests of cortical cells were occasionally observed. More frequently, segmental or diffuse hyperplasia of spindle cells extending from the adrenal capsule was seen (Fig. 7). Such subcapsular hyperplasia are common in mice of several strains (4).

Thyroid: Gross lesions were not found. Occasionally the thyroid was captured in sections of the upper respiratory tract. Small epithelial lined cysts, considered embryonal remnants of the ultimobranchial duct were found. The only other abnormality was distention of individual follicles that was diagnosed as follicular cysts.

Genitourinary System

Kidney: The most frequent finding was segmental or diffuse, usually minimal or mild glomerulonephritis. It was observed in both control and treated animals. The lesion was characterized by mesangial hypercellularity, sclerosis of glomeruli with adhesions to Bowman's membrane, fibrosis of Bowman's capsule, tubular casts and interstitial inflammatory infiltrates in severe cases (Fig. 8).

Agenesis and renal hypoplasia, considered as congenital malformations, were not unusual findings. Hypoplastic kidneys were characterized by marked cortical depletion with only atrophic glomeruli and occasional nests of normal appearing cortical tubules remaining (Fig. 9). Plasmacytic and lymphocytic infiltrates in the medulla were intense in some cases.

Other renal lesions included cortical cysts, hydronephrosis, perivascular inflammatory infiltrates, nephrocalcinosis, pyelonephritis and hyperplasia of the pelvic epithelium.

Ovary: Amyloid-like deposits, an infrequent finding, were found in areas of the ovary corresponding to corpora lutea (Fig.10). This may represent a genetic susceptability in the SENCAR as it is common in CD-1 mice (5).

The appearance of ovarian cysts or hematocysts are exceedingly common. The majority resulted from distension of the ovarian bursa with clear, tan or red fluid. Less frequently Graffian follicles or corpora lutea were cystic. The origin of the cyst was usually not identifiable. Microscopically a portion or the entire ovary may be obliterated with a single or multiloculated cyst (Fig. 11). Cysts often contained clear fluid or were blood-filled. The ovarian stroma surrounding the cyst was frequently fibrotic and heavily pigmented with blood breakdown products.

Uterus: Distention of a segment or both uterine horns was a common gross observation. Endometrial glands in such cases were hyperplastic and cystic (Fig. 12). Varying degrees of stromal hyperplasia accompanied the cystic hyperplasia.

Integumentary System

Clitoral Gland: Enlargement of the clitoral gland was a common gross finding in treated and nontreated mice and was caused by dilatation, inflammation or abscessation of the gland (Fig. 13). Neoplasms were not observed in our studies.

Mammary Gland: Inflammation and duct ectasia were common near skin paint sites.

Skin: Nontreatment related inflammatory lesions were frequently observed. They were usually of mild severity, consisted of slight inflammatory cell infiltration of hair follicles or were associated with hair or vegetable foreign bodies in the dermis (Fig. 14). Epithelial hyperplasia, erosion, ulceration and active chronic inflammation were common at or near skin paint sites . These provide difficult diagnostic decisions as regenerative attempts on the part of the host need to be differentiated from neoplastic changes. A more complete description of such lesions has been reported (6).

Hematopoietic System

Lymph Nodes: Lymphocytic and plasmocytic hyperplasias were the most common cause of enlarged lymph nodes. Granulomatous inflammation was observed in nodes near areas of chronic or active chronic inflammation. The cortical architecture of involved lymph nodes was obliterated by hyperplastic lymphoid tissue traversed by prominent bands of histiocytes and juvenile connective tissue. Medullary sinusoids were dilated with histiocytes.

Spleen: Hematopoietic cell hyperplasia (granulocytic and erythrocytic) and lymphoid hyperplasia were common causes of splenic enlargement. Such reactions were common in tumor-bearing mice and in mice with inflammatory processes elsewhere in the body. When the granulocytic hyperplasias are florid, their differentiation from myelogenous leukemia may be necessary (7). Amyloid-like deposits often were associated with the lesions. They involved the peripheral portion of splenic follicules and extended into the red pulp (Fig. 15).

Other incidental findings include focal fibrosis of the spleen and accessory splenic tissue usually in the mesentary near the pancreas.

Respiratory System

Lung: Inflammatory reactions included perivascular and peribroncial lymphocytic and plasmocytic infiltrates, and focal or diffuse areas of chronic, or active chronic inflammation. At times they appeared as small granulomas. Lesions in some cases had a propensity for terminal bronchioles and adjacent alveoli. Peribronchiolar alveolar lining cells were hypertrophied and interstitial areas were thickened by connective tissue proliferation and inflammatory cell infiltration (Fig. 16). Also observed were small focal collections of histiocytes usually located in alveoli near the pleura. These histological features were difficult to differentiate from those described for late stage Sendai virus infection (8,9).

Focal alveolar cell hyperplasia felt to be a precursor lesion to alveolar/bronchiolar neoplasms were infrequently encountered (Fig. 17).

SUMMARY

Ovarian cysts, cystic endometrial hyperplasia, and glomerulonephritis of a mild severity were the most frequently encountered lesions in this survey. An apparent low incidence level of a wide variety of other naturally occurring lesions were found to involve several organ systems.

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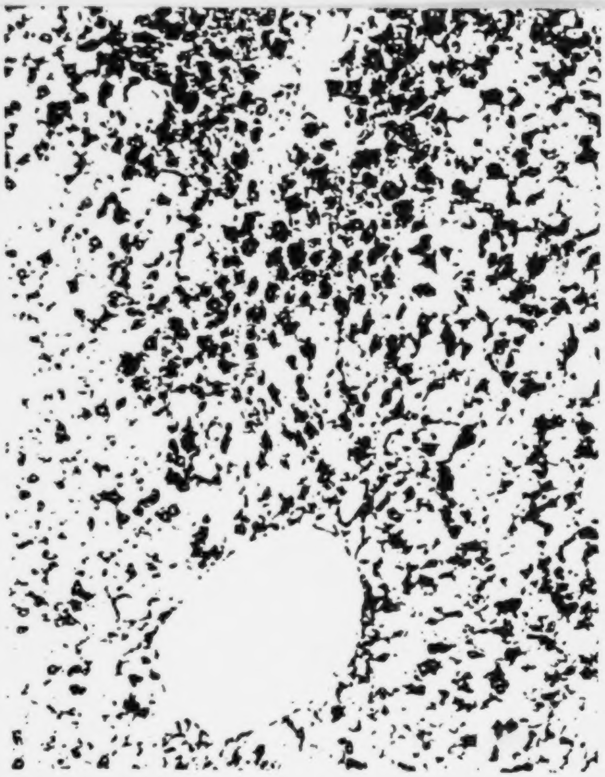

FIGURE 1. Amyloid-like deposits primarily involving peripheral portions of the liver lobule. (H&E, x 260)

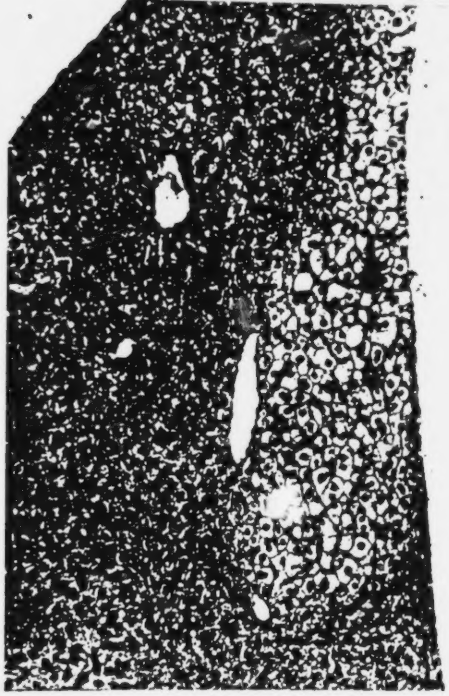

FIGURE 2. Focus of vacuolated hepatocytes located near the capsule of the median liver lobe. (H&E, 130)

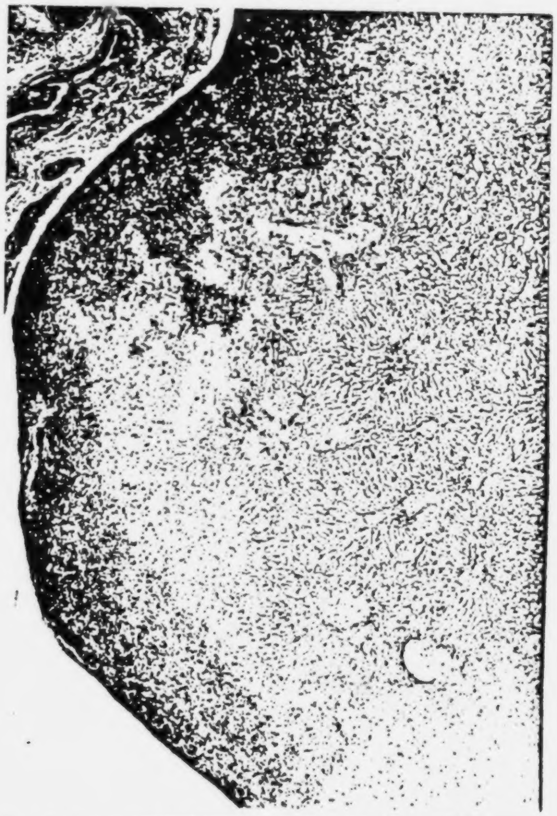

FIGURE 3. Ischemic liver necrosis centrally, surrounded by a connective tissue proliferation at the capsular surface, secondary to strangulation. (H&E, x 120)

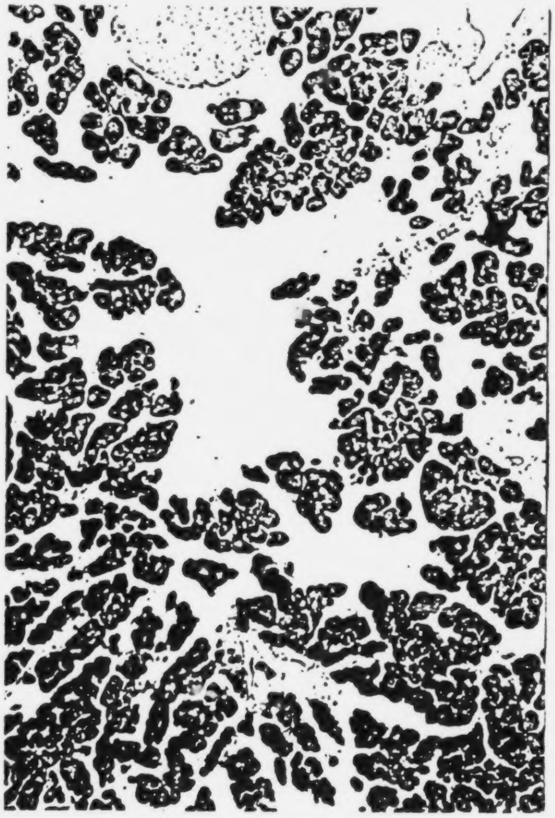

FIGURE 4. Acini depleted of zymogen granules in a pancreas described as being white grossly. (H&E, x 120)

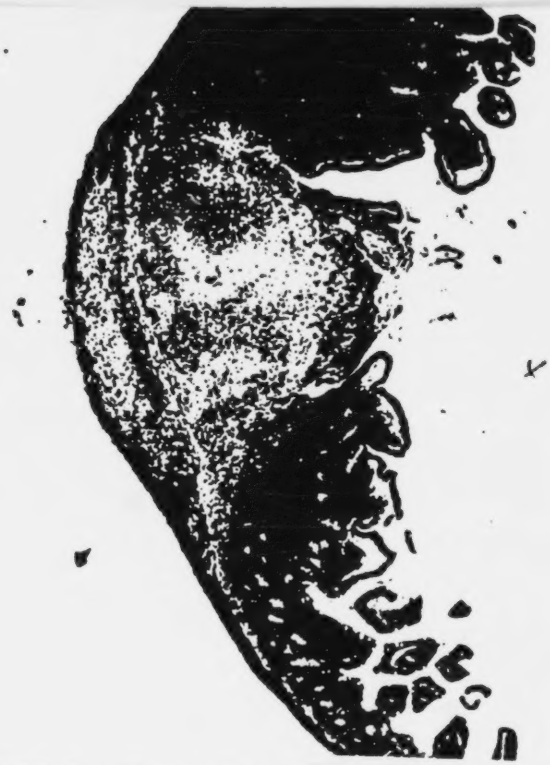

FIGURE 5. Ulcerated mucosa of the ileum. The serosa is thickened by inflammatory cell infiltration and connective tissue proliferation. (H&E, x 52)

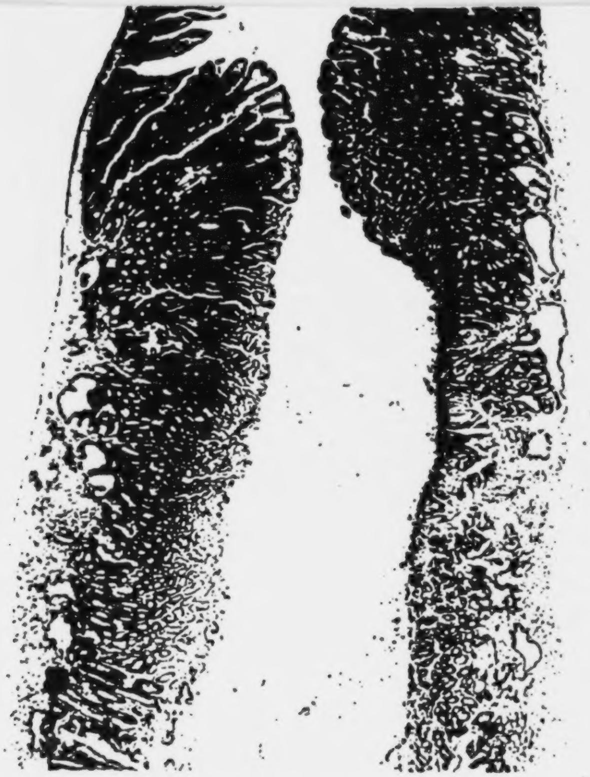

FIGURE 6. Segmental mucosal hyperplasia of the duodenal mucosa. Glands are ectatic near the base (H&E, x 26)

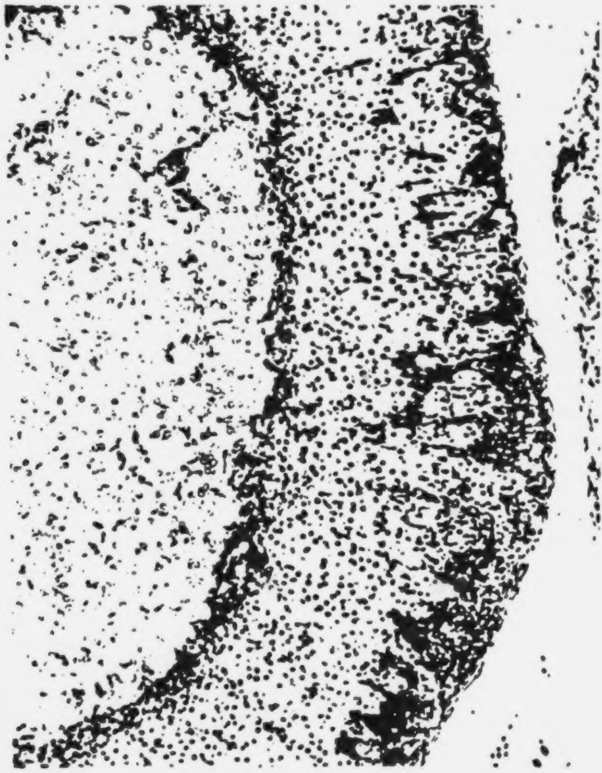

FIGURE 7. Subcapsular cell hyperplasia of the adrenal with nets of hyperchromatic cells extending to the zona fasciculata. (H&E, x 120)

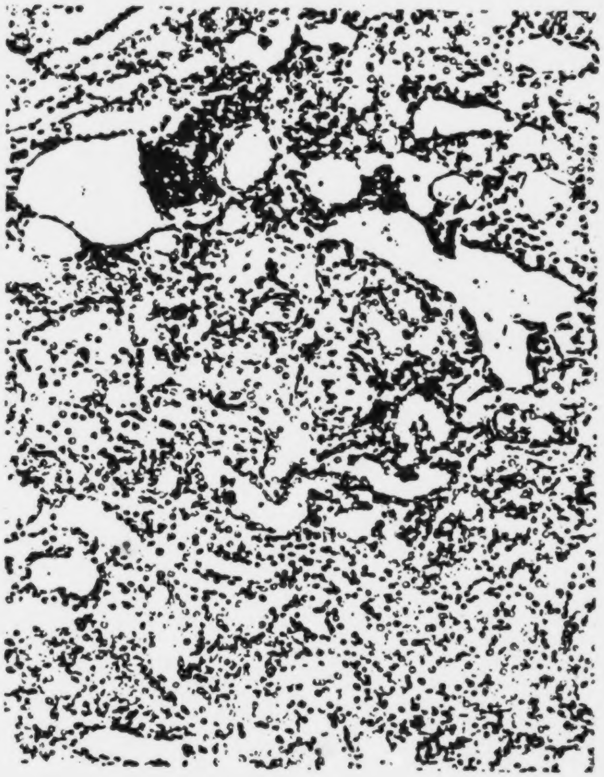

FIGURE 8. Sclerotic glomeruli, tubular casts and perivascular inflammatory infiltration in kidney. (H&E, x 260)

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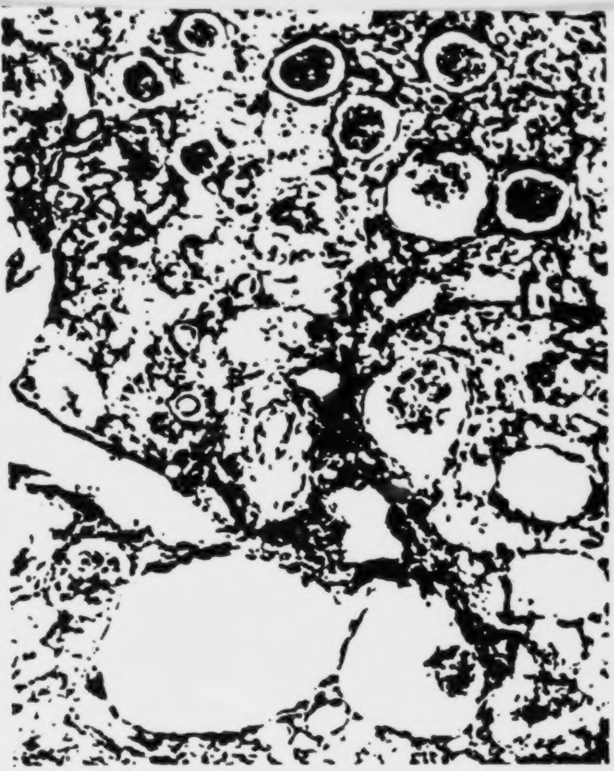

FIGURE 9. Kidney with marked cortical atrophy. A small nest of more normal appearing renal tubules is located near the top. (H&E, x 260)

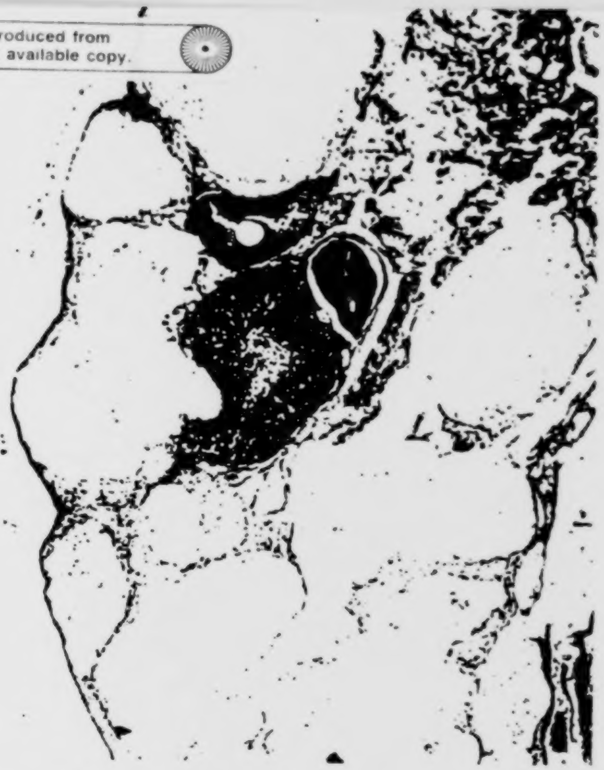

FIGURE 10. Amyloid-like deposits in the ovary. The principal deposits are in the corpora lutea. (H&E, x 52).

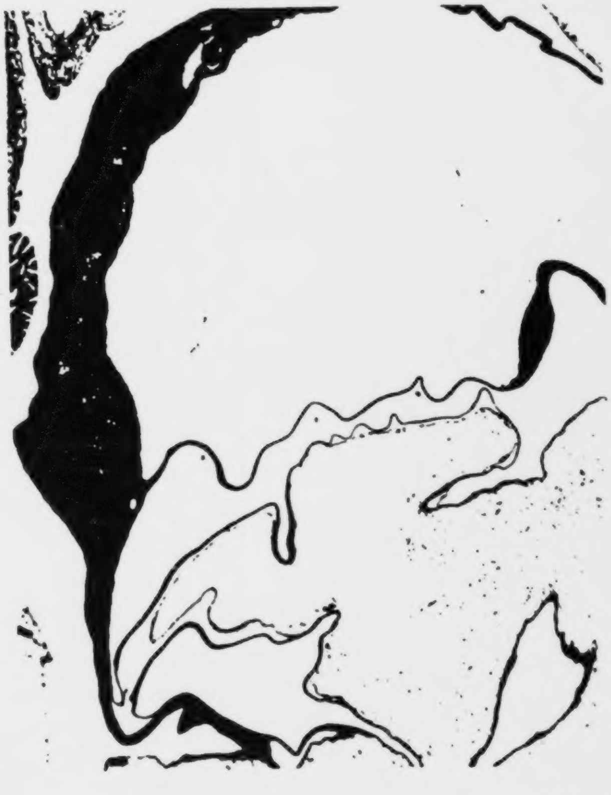

FIGURE 11. Ovarian tissue essentailly replaced by a multiloculated cyst. (H&E, x 52)

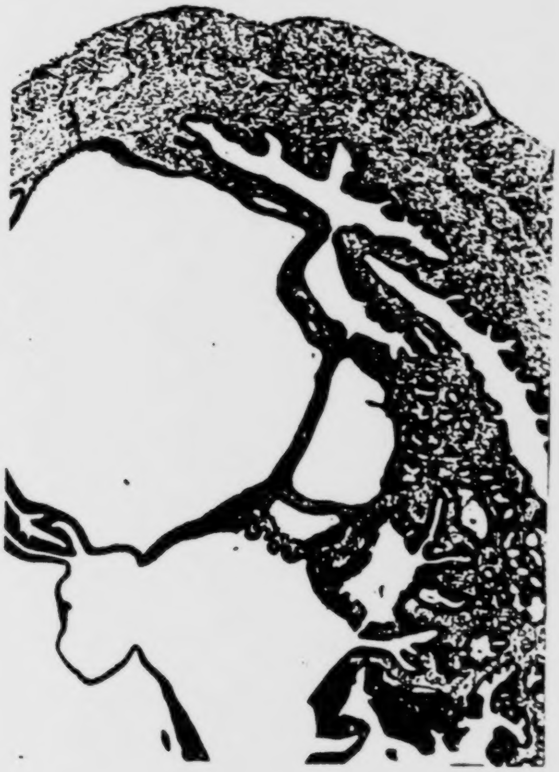

FIGURE 12. Cystic endometrial hyperplasia of the uterus. (H&E, x 26)

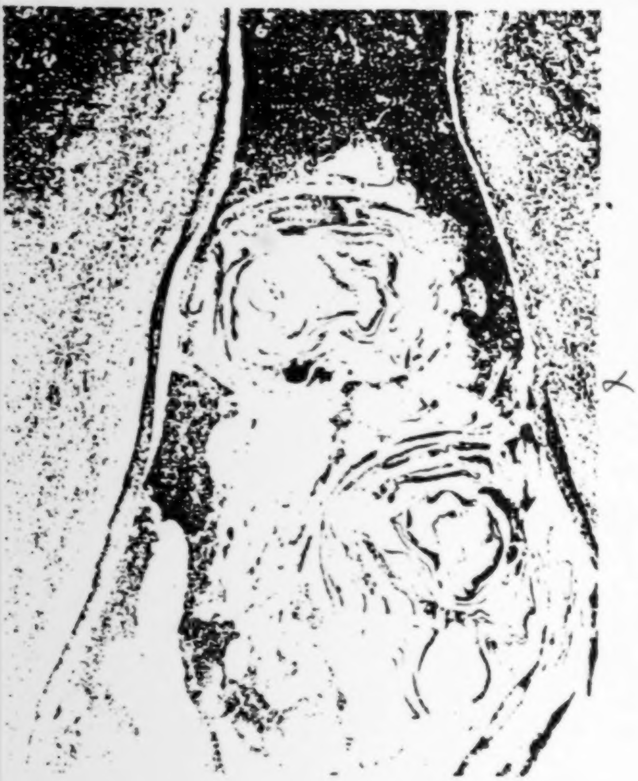

FIGURE 13. Inflammation and abscessation of the clitoral gland. The gland is distended with keratinacerous debris and purulent exudate. (H&E, 52)

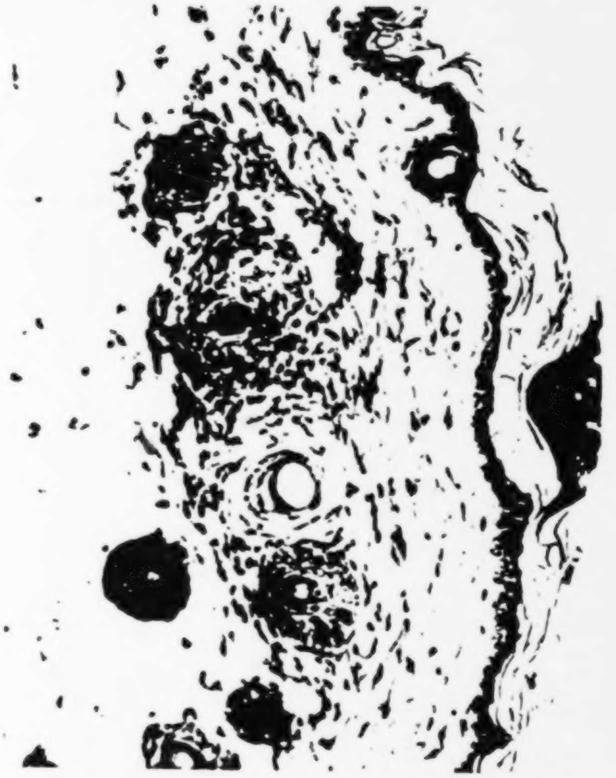

FIGURE 14. Perifollicular inflammatory infiltration involving the dermis. A small quantity of exudate is located on the epidermis. (H&E, x 260)

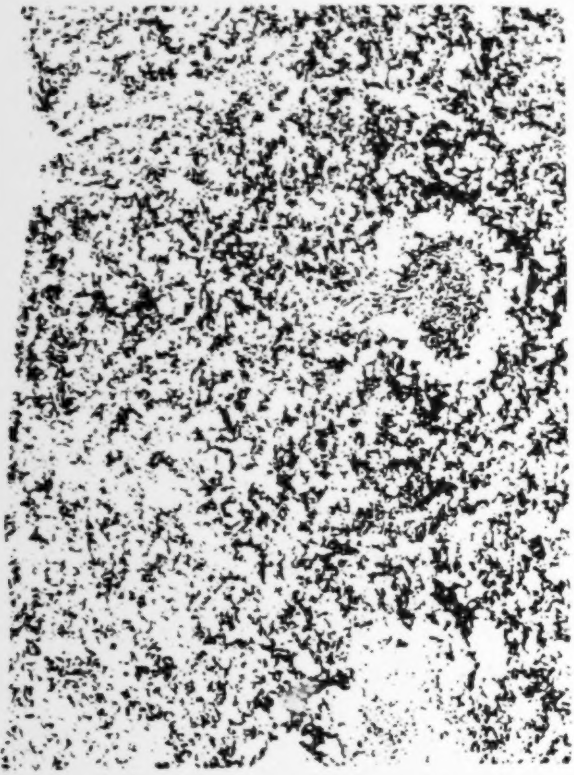

FIGURE 15. A spleen with perifollicular deposits of amorphous amyloid-like material. The red pulp is hypercellular due to hematopoietic cell proliferation. H&E, x 260)

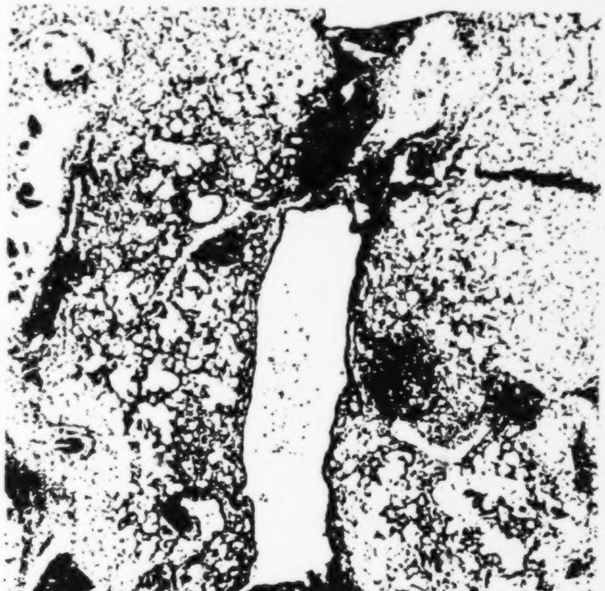

FIGURE 16. Peribronchial epithelial cell hypertrophy and hyperplasia, collections of macrophages in alveoli and perivascular and peribronchial lymphoid hyperplsia in the lung. (H&E, x 52)

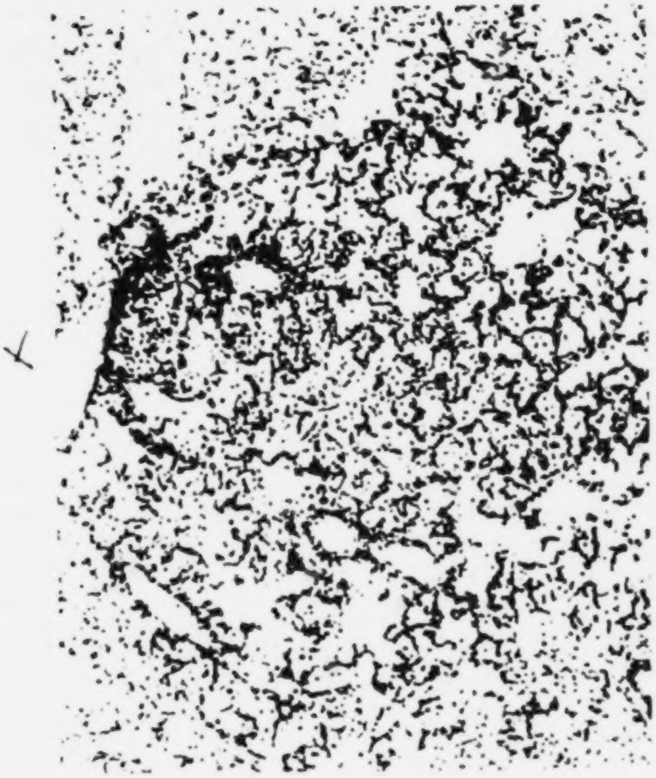

FIGURE 17. Hypertrophy of alveolar epithelial cells that rest on preexisting alveolar walls. Compression of adjacent pulmonary tissue is not significant. (H&E, x 260)

MYELOID HYPERPLASIA IN THE SENCAR MOUSE:
Differentiation from Granulocytic Leukemia

by

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KEY WORDS

1. MYELOID HYPERPLASIA
2. EXTRAMEDULLARY HEMATOPOIESIS
3. MYELOID METAPLASIA
4. LEUKEMOID RESPONSE
5. GRANULOPOIESIS
6. GRANULOCYTIC LEUKEMIA
7. MYELOID LEUKEMIA
8. MOUSE

ABSTRACT

The term myeloid hyperplasia has been used interchangably with many other terms to describe an increased production of granulocytes in the spleen and other organs in the mouse. This process is occasionally misdiagnosed as granulocytic leukemia. This paper reviews some of the terms used interchangably with myeloid hyperplasia and describes criteria which can be used in differentiating myeloid hyperplasia from granulocytic leukemia. Additionally, the results of a study in which myeloid hyperplasia was induced following the formation of skin tumors is discussed. In this study, positive correlations were found between skin lesions, the spleen weight and histologic appearance of the spleen. The liver rarely showed microscopic changes of myeloid hyperplasia unless the spleen weighed at least 1.0 percent of the body weight.

INTRODUCTION

Many strains of mice, including the Sencar stock, respond to inflammatory stimuli, especially of the skin, by greatly expanding the granulocyte pool with an increased production of granulocytes in various organs of the hematopoietic and lymphoreticular systems. Frequently, proliferation of granulocytic cells is accompanied by erythropoiesis, megakaryocytosis, plasmacytosis, and reticuloendothelial cell hyperplasia in various organs of these systems.

Many terms, such as myeloid hyperplasia, leukemoid response, myeloid metaplasia, extramedullary hematopoiesis and granulopoiesis have been used interchangably to describe this phenomenon. However, in their pure form all these terms are not synonymous. For instance, the term leukemoid response refers to an extreme elevation of the white blood cell count in the peripherial blood(1,2,3). Myeloid metaplasia and extramedullary hematopoiesis (EMH) is the formation and development of blood cells outside the bone marrow(1,3) and granulopoiesis is the formation of granulocytes(1,3). Myeloid hyperplasia, as we use the term, indicates an extreme increase in the formation and development of granulocytes above that level which is considered to be normal and is almost always associated with an inflammatory reaction. The use of these terms interchangably often results in confusion among those trying to describe and record similar lesions.

The problem encountered in trying to diagnose myeloid hyperplasia is compounded by the fact that microscopically observed lesions may mimic those of granulocytic leukemia(1,4,5,6,7). Although relatively rare when compared to neoplasms of lympoid cells, granulocytic leukemia does occur and must be differentiated from hyperplasia of granulocytic cells(4,9). The importance of separating myeloid hyperplasia from granulocytic leukemia can not be over emphasized. Many times the animals in which the hyperplastic or neoplastic cells are seen have been given a variety of test materials of potentially significant environmental impact. Misdiagnosis under these circumstances could have a profound effect on the outcome of the study and the compounds involved.

In this report we will attempt to clarify some of the terminology, report the results obtained from examination of cases on file and provide some data from a study designed to induce myeloid hyperplasia

MATERIALS AND METHODS

Reports from three studies on file at the Health Effects Research Laboratory/Toxicology and Microbiology Division (HERL/TMD) at the Environmental Protection Agency (EPA) were examined. Slides were evaluated from eight animals which had been diagnosed as having myeloid (granulocytic) leukemia, leukemoid reaction, extramedullary hematopoiesis, and/or granulopoiesis in one or more of the following organs: liver, spleen and lymph node.

Also, a study, using 160 Sencar mice divided into 4 groups of 40 animals each, was preformed to induce myeloid hyperplasia. Groups 1, 2, and 3 were initiated with one topical application of Benzo-a-Pyrene (2.0mg/kg). Promotion was started five days later with topical application of 12-O-tetradeconoyl-phrobol-13-acetate (TPA) 3 times a week for 15 weeks (7 weeks at 2.0μg TPA in 0.2ml of acetone followed by 8 weeks of 4.0μg TPA in 0.2ml of acetone). Group 4 was treated topically with 0.2ml of acetone at the time of initiation and following the same schedule as used for the promotion of groups 1 thru 3. During week 19 the mice in group number 2 were anesthetized with ether and up to 2 tumors per animal were removed. The incisions were closed and the mice were allowed to recover. During the same week the mice from group number 1 and half of the mice from group number 4 (group number 4ES) were sacrificed. The weights of the body, liver and spleen were recorded and selected tissues were placed into 10% buffered neutral formalin (BNF) for subsequent microscopic examination. During week number 29 the remaining mice in groups 2, 3 and 4 (group number 4LS) were sacrificed and the weights of the body, liver and spleen were recorded and selected tissues were placed into 10% BNF for subsequent microscopic examination. Tissues were processed, embedded in paraffin, sectioned at 5-6 microns and stained with hematoxylin and eosin for microscopic examination.

RESULTS

No reports of granulocytic (myeloid) leukemia in the Sencar mouse were found during our review of the literature. Based on published articles on other strains of mice, documented reports of spontaneously occuring granulocytic leukemia appear to be quite uncommon in the mouse(1,4,8,9). Even though it does appear to be rare, one must be aware of the criteria used in distinguising this condition from myeloid hyperplasia. However, even when these criteria are used many times differentiation is not easy.

In her extensive review of the hematopoietic and lymphoreticular systems Dunn details the criteria used to differentiate granulocytic leukemia and myeloid hyperplasia, referred to as "nonmalignant extramedullary myelopoiesis" in her article. These criteria as well as those of other authors are outlined in table 1(1,4,5,7). The most important criterion appears to be the presence or absence of various stages of granulocyte maturation(1,4,5,7). However, in the mouse it is difficult to identify all phases of development and usually only three stages can be distinquished. The young or early forms are larger cells with basophilic cytoplasm with or without azurophilic granules and a round or early ring form nucleus with finely stippled chromatin. The old or mature form has a pale basophilic or neutrophilic cytoplasm with a ring form nucleus showing early indentation or fully developed segmentation and coarsely distributed chromatin. The intermediate forms includes all the remaining cell stages(1,5).

The criteria listed in table 1 were used during the evaluation of slides from a previous HERL/TMD study. Sections from spleens which had been diagnosed as granulocytic leukemia, leukemoid response or extramedullary hematopoiesis were examined. There was no outstanding feature present to distinguish granulocytic leukemia from the leukemoid response. In fact, the granuylocytic cell population of the spleen diagnosed as leukemoid response appeared to have the most immature cell population with the most uniform stage of development, characteristics usually associated with granulocytic leukemia. The spleens diagnosed as having EMH in general could be readily differentiated from those with granulocytic leukemia or leukemoid response. They usually had more erythropoietic foci and fewer numbers of granulocytes. However, one mouse with EMH was histologically very similar to the one diagnosed as leukemoid reaction except the former had a greater number of mature granulocytes. Based on previously stated criteria and definitions it appears that these cases should have been diagnosed as EMH or myeloid hyperplasia.

Liver sections diagnosed as having granulocytic leukemia, leukemoid response or granulopoiesis were also evaluated. Again there were no discernable differences between the livers of mice with leukemoid response and those with granulocytic leukemia. There was extensive involvment of the liver primarly at the tips of the lobes in all these mice. In two animals, one with granulocytic leukemia and one with leukemoid response, necrosis of hepatocytes was accompanied by severe infiltration of granulocytes. However, in all instances, all stages of granulocyte development were present as were a few megakaryocytes scattered in sinusoids. Occassionally, small erythropoietic foci were present in livers of mice with leukemoid response. A large number of mature and a very few immature granulocytes were found within the lumens of blood vessels in the liver of one mouse diagnosed as having granulocytic leukemia. When evaluated alone the changes in one one of these livers may lead one to make a diagnosis of granulocytic leukemia. However, when considered along with changes seen in the rest of the animal a diagnosis of granulocytic leukemia may not be appropriate.

The livers of mice diagnosed as granulopoiesis were less severly affected and contained scattered foci of granulopoiesis in sinusoids and adjacent to blood vessels. Occassionally small foci of erythropoiesis were present but megakaryocytes were not consistently found. The granulocytes present in the livers were a mixture of all stages of granulocyte development. The major difference between these livers and those described previously was the number of cells and the amount of hepatic tissue involved.

The data from our study designed to induce myeloid hyperplasia showed a difference among the groups when the mean spleen weights per group, expressed as a percentage of the body weight, were compared (Fig1). The mean spleen weight of group number 1, the treated group sacrificed early, and group number 3, the treated group sacrificed at the end of the study, were essentially the same. Group 4ES, the half of the vehicle control group sacrificed early, and group 4LS, the half of the vehicle control group sacrificed at the end of the experiment, were essentially the same and both were much lower than groups 1 and 3. The mean spleen weight of group number 2, the treated group with the tumors surgically removed and sacrificed at the end of the study, was lower than groups 1 and 3 but slightly higher than groups 4ES and 4LS. When the same comparisons were made using the liver weight expressed as a percentage of the body weight, no difference was found among any of the groups (fig 1).

When the mean liver and spleen weights, expressed as a percentage of the body weight, were evaluated based upon the number of skin tumors and the presence of dermal ulcerations not associated with skin tumors a definate pattern was seen (fig 2). (The number of skin tumors was used as a measure of inflammation. In general, as the number of tumors increased so did the amount of inflammation associated with the tumors.) There was a gradual increase in the mean weight of the liver and spleen as the number of tumors increased. The liver and spleen weights were highest when dermal ulcers, not associated with skin tumors, were present. The increases in liver and spleen weights parallel each other in mice which only had skin tumors. In mice that had dermal ulcerations, not associated with tumors the differences between the liver and spleen weights was much greater. This difference was attributable to the greater increase in liver weight.

Weights of the spleens appeared to increase as a result of increased number of cells of the erythrocytic and granulocytic cell lines in the spleen. Usually this was not detected microscopically until the spleen weighed 0.5 to 0.7 percent of the body weight. In contrast to the spleen, the mild increases in the weight of the liver were not as readily attributed to cells of the granulocytic or erythrocytic cell lines and in many cases no histologic evidence for these increases were found. No granulocytic precusors were seen in the liver unless the spleen was also involved except in two instances. In most cases the spleen was severely affected and accounted for greater than 1.00 percent of the body weight before the liver was involved. Pathological changes in the lymph nodes were not observed unless the spleen was severely infiltrated by granulocytic cells.

At necropsy, the spleen and lymph nodes were consistently enlarged in animals which had a large number of tumors or had dermal ulcerations not associated with skin tumors (figs 3 and 4). Microscopically in the spleen, granulopoietic foci first appeared just beneath the splenic capsule and adjacent to the trabeculae (fig 5). As the number of granulocytic cells increased these foci expanded and formed a rim of proliferating cells beneath the capsule and around the trabeculae (fig 6). In severe cases, granulocytes and their precursors began filling the red pulp (fig 7). In all instances, megakaryocytes and foci of erythropoiesis were scattered among the proliferating granulocytes throughout the red pulp. In the liver, foci of granulopoiesis were found adjacent to hepatic and portal vessels as well as in sinusoids which occassionally contained megakaryocytes (fig 8). The reaction in the lymph node was characterized by extensive plasma cell infiltration of the medulla with or without granulocytes (fig 9). Reticuloendothelial cell hyperplasia was an inconsistent finding.

DISCUSSION

Granulocytic leukemia and myeloid hyperplasia are not always easily distinguished from one another especially when only one or two tissues are available for microscopic examination. In granulocytic leukemia the cells are immature usually with one stage predominating. However, in myeloid hyperplasia all stages of development can be found(1,4,5,7). Other commonly used criteria for differentiating these two conditions are the presence of erythropoiesis, megakaryocytes and an inflammatory reaction which are typically found with myeloid hyperplasia but are usually not associated with in granulocytic leukemia(1,5,7). When the number of available tissues is limited, the presence of capsular invasion, the age of the mouse and the presence of hemorrhages should also be considered when making a diagnosis(1,5,7,9).

However, a thorough macroscopic and microscopic examination of a complete set of tissues is more likely to provide information which will enable one to separate these two conditions(5). Items which should be noted during the necropsy include: 1) the presence of inflammatory conditions, 2) the size of the liver, spleen and lymph nodes, 3) the weights of the liver and spleen and 4) presence or absence of hemorrhage. Tissues to be collected to facilitate a diagnosis include the spleen, liver, peripherial lymph nodes, bone marrow (formalin fixed and fresh smears), kidneys, adrenals, salivary glands, and ovaries. If the live animal is available additional techniques such as collection of blood for a complete blood count with a differential count and the harvesting of tissue for transmission and transplantation studies should be done(1,5,7).

The extent of organ involvment can only be determined if a full set of tissues is available. This information can be most useful in differentiatng these two conditions because the order in which the organs are affected is different in each condition. In myeloid hyperplasia, the bone marrow is one the first tissues to be affected. This is in contrast to granulocytic leukemia inwhich bone marrow involvement occurs late in the course of the disease and is associated with destruction of bone. Additionally, in granulocytic leukemia the spleen is the first organ affected followed by the liver. Neoplastic cells infiltrate the lymph nodes, bone marrow and other organs during the later stages of the disease(5,7,9).

The study we conducted was designed so that no tumors or dermal ulcerations would to be present on any animal in group number 2 at the end of the experiment. We discovered the amount of tissue that had to be excised to insure complete removal of the skin tumors was so great that no more than two tumors could be removed from any one mouse. Therefore, when evaluating of the group data for groups 1, 2, and 3 only those animals with one or two skin neoplasms were used.

The presence of the myeloid hyperplasia at week 19 and week 29 was reflected by the elevated mean splenic weight of group 1 and group 3 respectively. In both groups the increased mean spenic weight correlated microscopically with an increase in the number of cells of the granulocytic and erythrocytic cell lines. The absence of an increased mean splenic weight in the tumor free, vehicle control mice (group number 4) at the early and the terminal sacrifice suggests that the presence of skin tumors provided the stimulus for the induction of the myeloid hyperplasia. The low mean splenic weight of group number 2 at the terminal sacrifice suggests that myeloid hyperplasia was reversed by the removal of the tumors. The lack of correlation between increased mean liver weight and histologic evidence of myeloid hyperplasia in the liver in mice having 1 or 2 skin tumors may have been due in part to the low level of liver involvement or the lack of uniform changes within the lobes of the liver.

As the number of skin tumors increased, there was a trend toward greater liver and spleen weights. The marked rise in liver and spleen weights when dermal ulcerations, not associated with skin tumors, were present suggests the accompanying inflammatory reaction was a positive stimulus for the initiation of myeloid hyperplasia. Increased production of granulocytes as a result of an inflammatory lesion is well documented in the literature(2,7,10). Increased spleen weights in mice with dermal ulcerations correlated microscopically with the presence of myeloid hyperplasia. This finding was to be expected based upon reports in the literature(7). However, the greater increase in the liver weight when compared to the spleen weight in the presence of dermal ulcerations was not expected and is not easily explained.

In conclusion, myeloid hyperplasia usually results from an increased demand for granulocytes and may affect the spleen, liver, lymph nodes as well as other organs. It is most commonly induced by inflammatory conditions. Histologically, the spleen, liver, lymph nodes and occassionally other organs may be infiltrated by proliferating granulocytes that on occassion can be mistaken for neoplastic cells associated with granulocytic leukemia. A number of microscopic criteria can be applied to help distinquish between the two conditions. However, they are much easier to differentiate if a complete diagnostic work up is performed.

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Table 1
Myeloid hyperplasia vs granulocytic leukemia in the mouse

Myeloid Hyperplasia	**Granulocytic Leukemia**
All stages of granulocyte development present	Usually immature cells with one stage predominating
Erythropoietic activity usually present	Erythropoietic activity absent
Megakaryocytes are usually numerous	Magakaryocytes are few and present in organs where they are normally found
Frequently associated with inflammation	Not always accompanied by inflammation
Cells are not invasive	Cells are invasive
Normal tissue of the spleen is relatively unaffected	Normal tissue of the spleen is extensively replaced
Blood is normal or leukocytosis with mature forms	Blood usually contains immature forms
Hemorrhages are absent	Hemorrhages are frequent
Not transmissible	Transmissible
Not transplantable	Transplantable
Can occur at any age	Usually occurs in mice over 12 months old
When the lymph nodes are involved the cells are usually confined to the medulla	When the lymph node is involved the cells are not confined to the medulla
Bone marrow is involved late	Bone marrow is involved early
Bone marrow involvment does not result in destruction of the bone	Bone marrow involvment results in destruction of the bone

Figure 1. Spleen and Liver weights by group. Spleen and liver weights expressed as a percentage of body weight compared by groups.

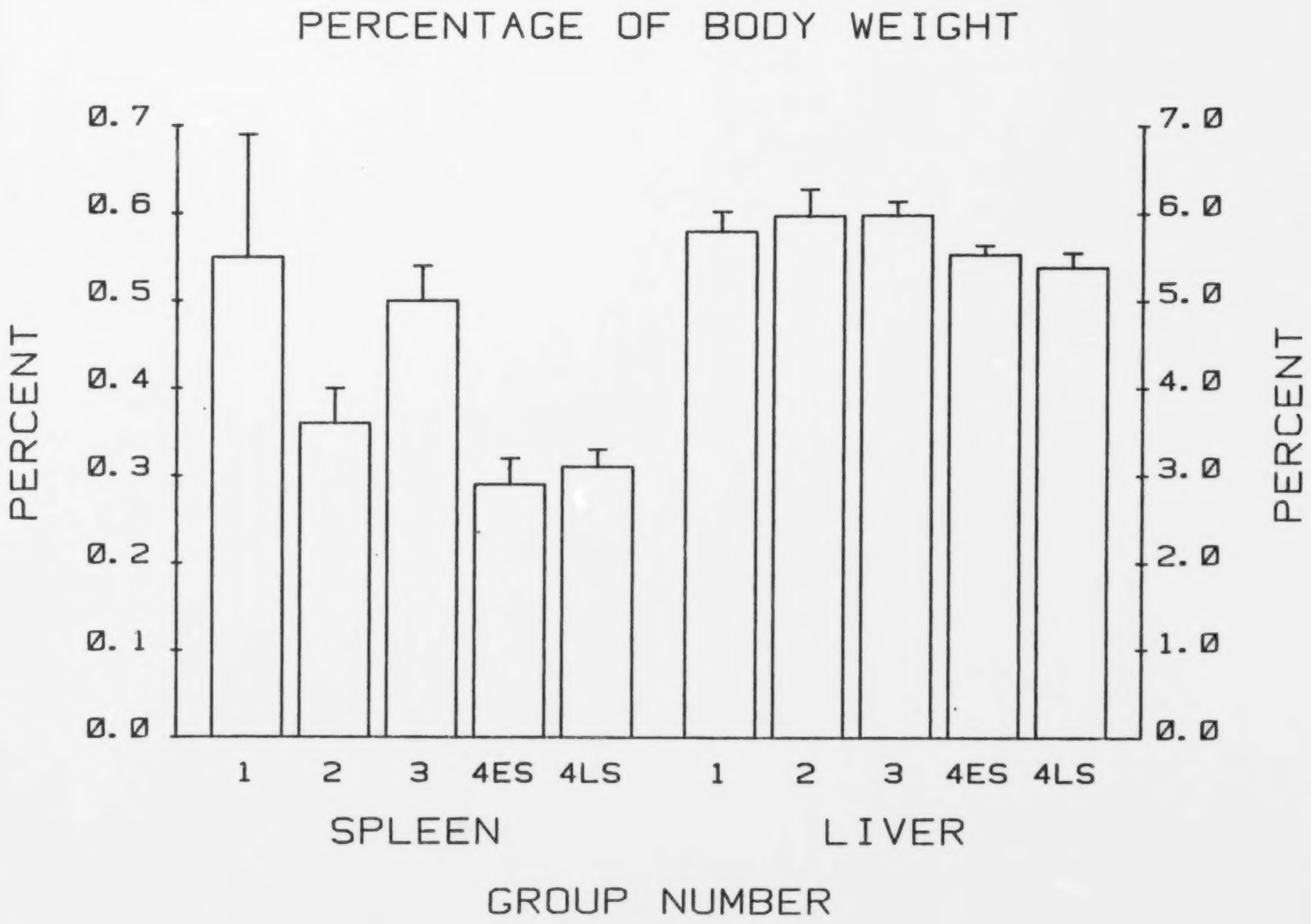
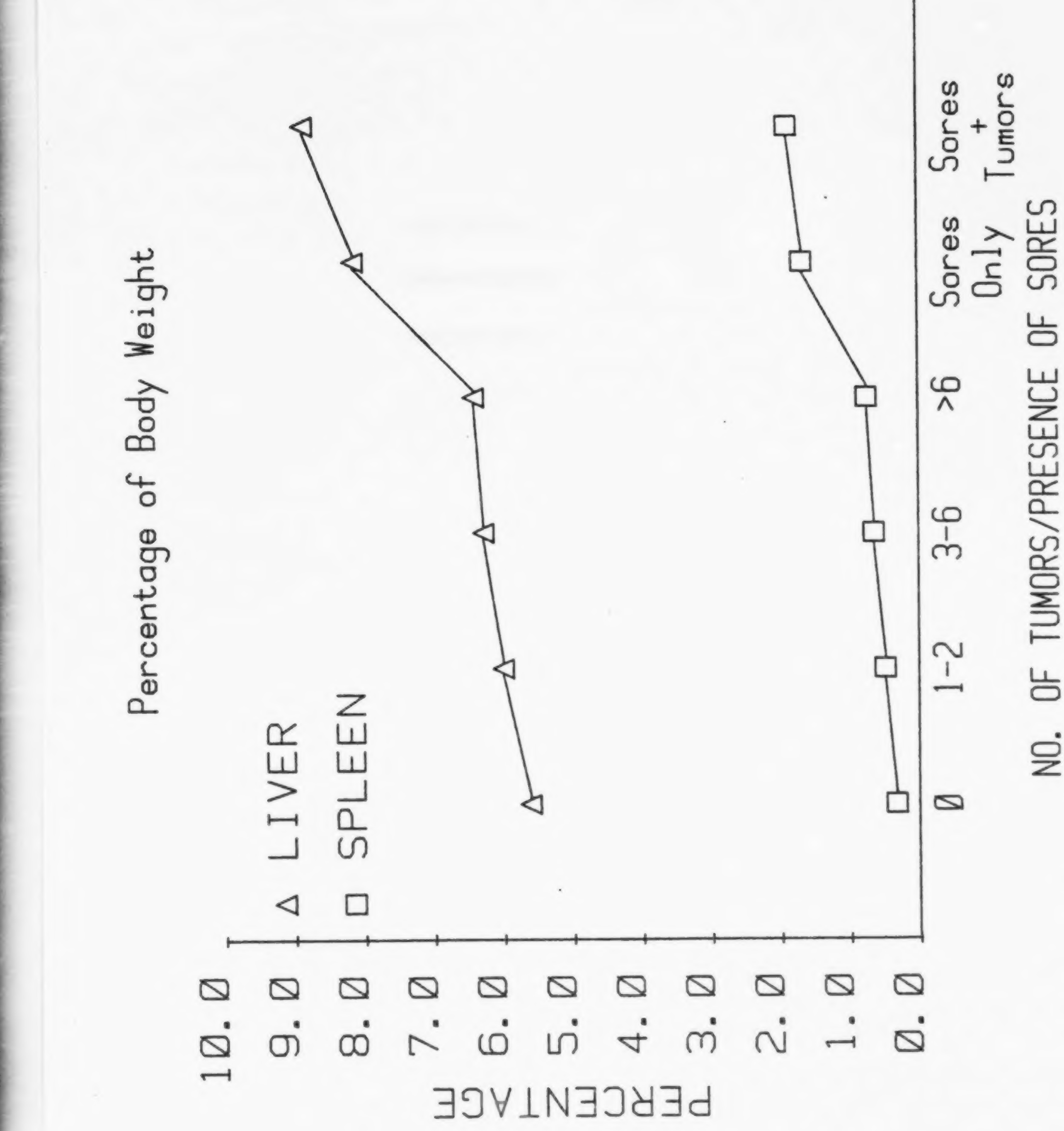

PERCENTAGE OF BODY WEIGHT
PERCENT
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PERCENT
1
2
3
4ES
4LS
1
2
3
4ES
4LS
SPLEEN
LIVER
GROUP NUMBER

Figure 2. Spleen and liver weights by skin lesions. Spleen and liver weights expressed as a percentage of body weight compared by the number of skin tumors present and the presence of dermal ulceration not associated with skin tumors.


Percentage of Body Weight
△ LIVER
□ SPLEEN
PERCENTAGE
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
1-2
3-6
>6
Sores Only
Sores + Tumors
NO. OF TUMORS/PRESENCE OF SORES

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best available copy.

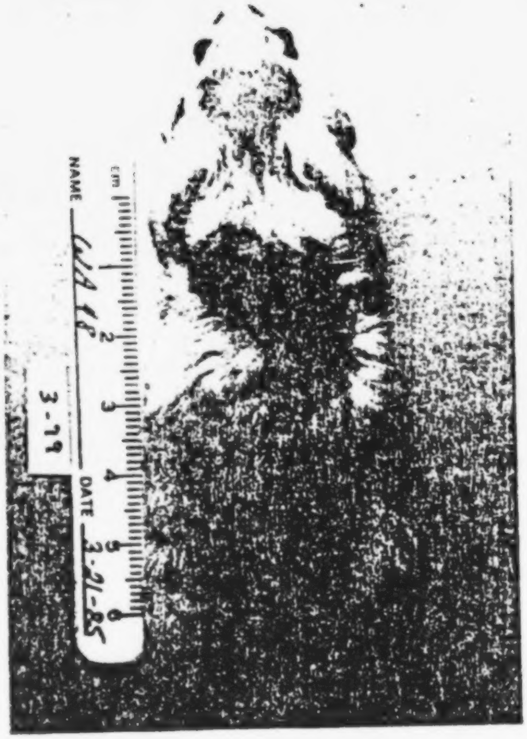

Figure 3. Dermal ulceration. Note the large area of dermal ulceration over the anterior one third of the back.

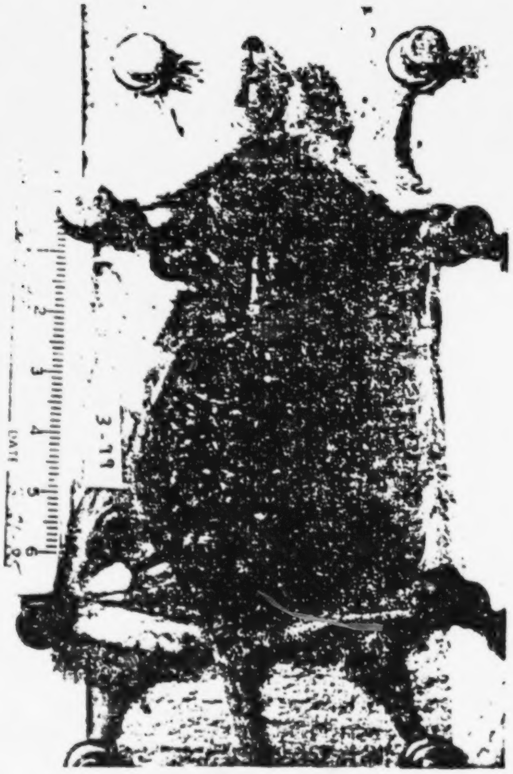

Figure 4. Enlarged spleen and lymph nodes. Note the enlargment of the spleen and peripheral lymph nodes.

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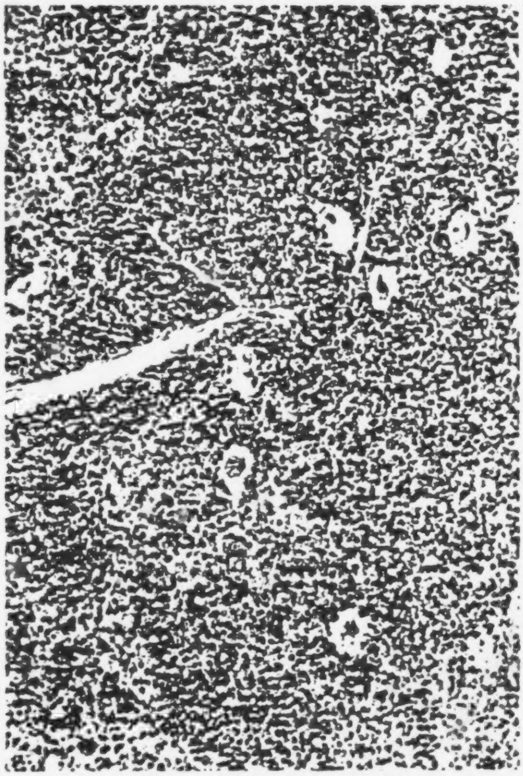

Figure 5. Mild myeloid hyperplasia in the spleen. Note granulopoietic foci adjacent to trabeculae, megakaryocytes and foci erythropoiesis.

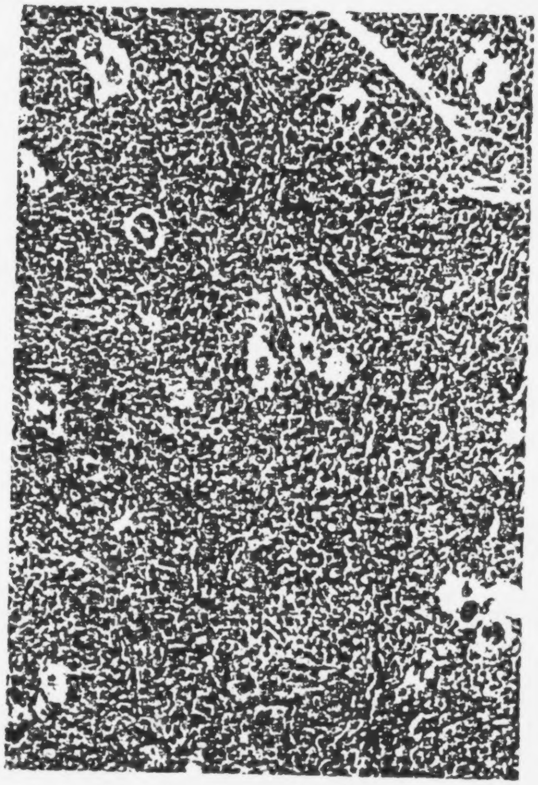

Figure 6. Moderate myeloid hyperplasia in the spleen. Note the rim of proliferating granulocytic cells adjacent to trabeculae, megakaryocytes and foci of erythropoiesis.

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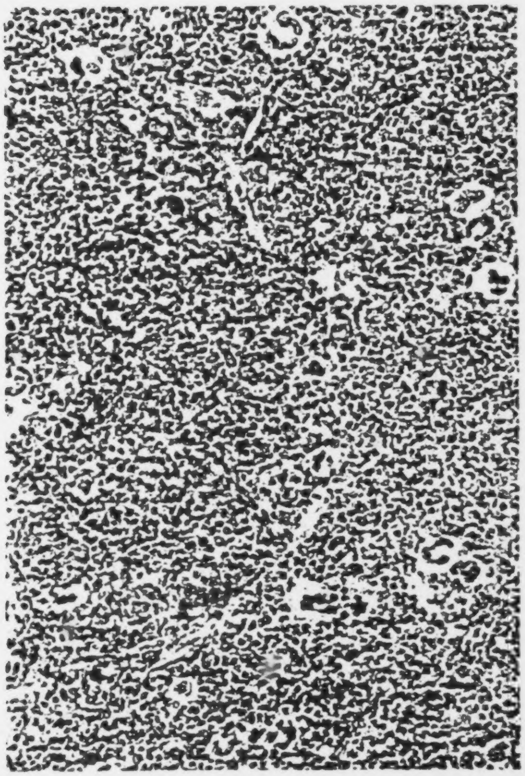

Figure 7. Severe myeloid hyperplasia in the spleen. Note diffuse granulopoiesis involving the red pulp, megakaryocytes and foci of erythropoiesis.

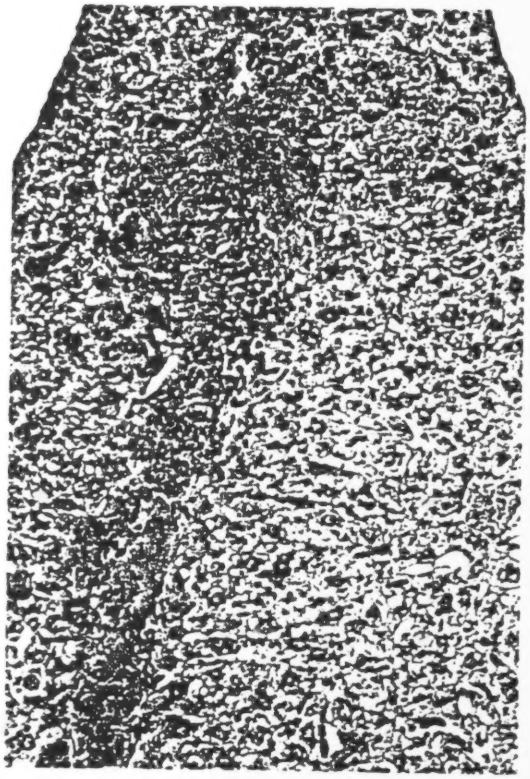

Figure 8. Myeloid hyperplasia in the liver. Note foci granulopoiesis adjacent to vessels as well as in sinusoids.

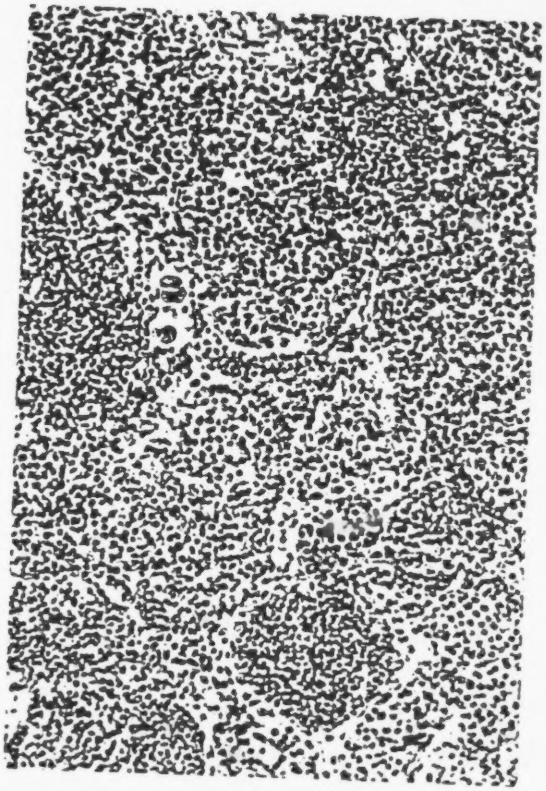

Figure 9. Myeloid metaplasia and plasmacytosis in a lymph node. Note the severe infiltration of the lymph node by plasma cells. Scattered foci of granulopoiesis and megakaryocytes are also present.

A COMBINED CARCINOGEN BIOASSAY UTILIZING BOTH THE LUNG ADENOMA AND SKIN PAPILLOMA PROTOCOLS

by

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KEYWORDS: carcinogenesis, strain comparison, skin papilloma, lung ademona

ABSTRACT

To test the feasibility of employing a combined lung adenoma/skin papilloma assay for broader detection of chemical carcinogenesis than realized with either bioassay done separately, four strains of mice; SENCAR, BALB/c, A/J and ICR-Swiss, were administered carcinogens by either the oral or I.P. routes. The carcinogens administered were ethyl carbamate (EC), benzo(a) pyrene (BP), N-[4-(5-nitro-2-furyl)--thiazolyl]-formamide (FANFT), and acrylamide (ACR). Starting two weeks later, 1-5 υg (depending on strain) of 12-0-tetradecanoyl-phorbol-13-acetate (TPA) in 0.2 ml acetone/mouse was applied 3 times weekly to the shaved back for 20 weeks. All strains displayed increases in the yield of lung adenomas in response to EC at 32 weeks. BP increased lung adenomas in only the SENCAR and A/J strain. Only the SENCAR and ICR-Swiss mice gave positive responses in the skin. In the SENCAR this was seen with all of these 4 chemicals, however, FANFT gave an inconsistent response. The ICR-Swiss responded with an increased skin papilloma yield only to EC. In a separate experiment involving only SENCAR mice, animals were treated with a single oral dose of diethylnitrosamine (DEN) followed by tri-weekly application of 1.0 μg TPA. This treatment resulted in 51/57 animals developing lung adenomas vs. 5/57 in the control animals. No treatment related skin tumors resulted with DEN. Histopathologically confirmed lesions indicate that the spectrum of chemicals detected in the SENCAR mouse may be broadened using a combined bioassay that examines both lung and skin responses.

INTRODUCTION

The mouse skin papilloma assay as described by Berenblum _et al._ (1,2) and Boutwell _et al._ (3,4) and mouse lung adenoma assay as described by Shimkin _et al._ (5) and Stoner _et al._ (6) are two carcinogen test systems that have been suggested by Bull _et al._ (7) for Tier 2 confirmation testing of putatively carcinogenic chemicals or mixtures. Although several different strains of mice have been utilized for these tests, the SENCAR mouse is the most sensitive model in the skin papilloma assay (8) and the A/J strain is the most susceptible model for lung adenomas (9). In an attempt to offset the occurrence of false negatives in these two carcinogen test models, our laboratory is testing the feasibility of utilizing a single mouse model for conducting a combined assay for skin papillomas and lung adenomas. To that end we have studied skin and lung responses of four different strains of mice to the administration of four different chemical carcinogens. One strain (ICR-Swiss) was examined because it is known to be sensitive to the induction of both lung adenomas and skin papillomas by chemical carcinogens (9,10). The BALB/c strain was included as a strain that was recognized to be relatively insensitive to skin tumor initiation and lung tumor development. All four strains were compared grossly for their carcinogenic response to BAP, EC, ACR, and FANFT (DEN was also tested in the SENCAR) and these findings were more critically assessed by histopathological examination.

METHODS

Chemicals

BP was obtained from Aldrich, Milwaukee, WI, DEN from Eastman Kodak Co., Rochester, NY, FANFT from Saber Labs, Morton Grove, IL, and EC from MCB, Gibbstown, NJ. BP was designated as 98% pure and the other chemicals were of reagent grade classification and all were used as received. ACR was purchased from Eastman Kodak and found to be more than 99% pure by GC-MS analysis (11). TPA was purchased from Chemical Carcinogenesis, Eden Prairie, MN.

Biological Testing

Only female mice were used for this study. The SENCAR and ICR-Swiss were obtained from Harlan Sprague-Dawley, Inc., Indianapolis, IN, BALB/c from Charles River, Wilmington, MA, and A/J from Jackson Labs, Bar Harbor, ME. Three experiments were conducted. In a first experiment DEN was tested only in the SENCAR mouse. A single dose of 50 mg/kg was administered by oral intubation in a 10% emulphor (polyoxygenated vegetable oil) solution. There were 60 animals in the group with 40 receiving TPA (1.0 μg 3 times weekly for 20 weeks) and 20 receiving acetone as indicated above. The skin tumors were charted for one year in this group and lung adenoma determinations made following sacrifice at that point. In the second experiment only SENCAR mice were tested, and BP was administered in a single dose of 100 mg/kg, rather than the 50 mg/kg as used in experiment 3. All other aspects of the protocol are the same as described below for the third experiment. In the third experiment a single dose of EC (300 mg/kg), BP (50 mg/kg), or FANFT (500 mg/kg) was administered interperitoneally to 60 mice of each of the four strains. Two days following this dose 40 of the 60 mice in each group received 1.0 μg (SENCAR), 2.5 μg (A/J and ICR-Swiss), or 5.0 μg (BALB/c) TPA in 0.2 ml acetone applied topically 3 times weekly for 20 weeks. The remaining 20 mice received only acetone for the same schedule and duration of treatment. The appearance and regression of skin tumors were charted weekly. Only tumors of 1 mm or greater and persisting for at least 3 observations were included in the cumulative count. The animals were sacrificed at 36 weeks of age and gross lung adenoma counts were determined according to the method reported by Shimkin (5).

In each study papillomas were included in the tumor count only if they were observed for 3 consecutive weeks. At the time of sacrifice all gross lesions were noted and fixed in 10% buffered formalin for subsequent histopathological evaluation. To further validate the gross lung tumor counts in studies 2 and 3, all questionable lung lesions were examined microscopically and total counts were adjusted to reflect confirmed adenomas. Skin lesions present at sacrifice were also examined microscopically (findings not reported). To determine statistical significance of the data, the chi-square was used for experiment one. For experiments 2 and 3, lung adenoma data were analyzed by Fisher's Exact Test and skin papilloma data by the Gehan and Wilcoxon type multiple comparison test (12).

RESULTS

In the first experiment (Table 1) treatment with DEN resulted in a significant increase ($P < 0.0001$) in the number of mice bearing lung tumors compared to the vehicle control group. The Shimkin method of counting lung adenomas was not utilized in this experiment and, therefore, the total adenoma count is not available. Of the animals treated with DEN, only 13%

had skin papillomas while in the control group 23% of the animals developed papillomas. This high rate may be partially attributable to the longer time to sacrifice in this experiment, but still represents a higher rate than the average for historical controls for SENCAR mice held for the same period in our laboratory (11%). These data suggest that SENCAR mice might be appropriately used in a combined skin and lung assay.

The skin and lung responses of the four strains of mice following treatment with three known carcinogens are presented in Table 2. The relative sensitivity of the different mouse strains to EC-induced lung adenomas was A/J > SENCAR = ICR > BALB/c with the latter strain displaying a very marginal response. ACR also induced lung tumors, but the sensitivity of the SENCAR and A/J mice was approximately equivalent. A very marginal response was seen in ICR mice and no lung tumors were observed in BALB/c mice. BP produced an increased yield of lung tumors in the SENCAR ($P < 0.05$) and A/J ($P < 0.05$) strains. A slight increase in lung adenomas was seen in ICR-Swiss mice with exposure to BP but was not significant. FANFT was negative for lung adenomas in all strains. Both experiments 2 and 3 gave rise to similar results in the yields of lung adenomas in the SENCAR mouse.

Only the SENCAR skin responded positively to carcinogen administration (Table 2). The four chemicals used in the second experiment all increased the papilloma count in the SENCAR. In the third experiment somewhat smaller responses were observed in the case of BP that could be partially accounted for by the lower dose used in the third experiment. However, decreased responses in the third experiment to EC and FANFT appear to reflect variability in the model. ACR produced a small but consistent response in both the SENCAR and ICR strains which would ordinarily be considered negative. However, concurrent experiments at higher doses have clearly shown that acrylamide is capable of initiating skin tumors in both the SENCAR (11) and the ICR Swiss mouse (10). In the ICR-Swiss only EC and FANFT increased skin tumor yields but these were not significantly different from control.

Another experiment which employed the same dose of TPA for all strains (1 μg 3 times weekly) gave rise to similar lung responses to these chemicals (data not shown). However, at this dose of TPA only the SENCAR skin responded to the initiators.

In all animals for studies 1 and 3, lesions diagnosed as primary lung tumors by the Shimkin method and all other lung lesions of questionable diagnosis based on gross examination were evaluated microscopically for confirmation and final tumor counts were adjusted based on these findings. In the ICR-Swiss, BALB/c, and A/J strains, there was generally close diagnostic correlation between the Shimkin gross interpretation and the microscopic diagnosis. However, in the SENCAR, the presence of a variety of inflammatory and proliferative lesions in the lungs greatly compromised the diagnostic accuracy of the Shimkin method. The morphologic features of various lung lesions observed in the SENCAR are demonstrated in Figures 1 through 5. Typical subpleural primary lung adenomas were frequently found in the SENCAR lungs in this study (Figure 1). The lesion is morphologically solid in type, and on gross examination presents as a raised pale tan circumscribed nodule. However, nonneoplastic lesions in the SENCAR interfere with the accuracy of the Shimkin method of lung adenoma counting in that they often appear grossly similar to primary tumors. These lesions occur as chronic-active or granulomatous inflammation (Figure 2) and consist of alveolar lining cell hyperplasia, alveolar histiocytosis and interstitial fibrosis with infiltration of interstitial and alveolar spaces by lymphocytes (Figure 3). Lung tumor counting in SENCARs is further complicated by the size, depth, and loose arrangement of many adenomas which makes them less easily defined on gross examination (Figure 4) and by the presence of metastatic squamous cell carcinomas (Figure 5) that result from the carcinogenic response in the skin.

CONCLUSION

This study confirms the findings of Slaga *et al.* (8) that the SENCAR is the most sensitive of the four strains tested for use in the skin papilloma assay. In addition, we have shown that the SENCAR also responds to chemical carcinogens by the development of lung adenomas. However, the presence of inflammatory changes in the lungs that have a similar gross appearance as primary tumors makes it necessary to confirm microscopically all lung lesions in the SENCAR mouse. Although these proliferative inflammatory lesions may be treatment-related, it could not be determined whether they were induced by the crcinogens utilized, emulphor, or some other agent.

While the A/J strain is the most sensitive in the lung adenoma test it was totally non-responsive in the skin to the chemicals tested. The BALB/c mice were also negative for these chemicals in the skin and only very marginally responsive in the lung with EC and FANFT treatments. The carcinogenic activity of the three chemicals tested in ICR-Swiss mice gave rise to responses in both the lung and skin. However, the ICR-Swiss mice were generally less responsive than the SENCAR in both target organs.

It should be noted that the present study does not assess the responsiveness of the lung in the classical manner in that only one dose of carcinogen was administered. Repeated dosing as advocated in the original studies of Shimkin would increase the yield of lung adenomas. For example, repeated oral or intraperitoneal doses of 25 and 30 μg/kg of ACR 3 times weekly for 8 weeks respectively gave rise to approximately 1.4 and 2.2 tumors per animal in recent studies (11).

Ideally, the application of a combined bioassay should utilize the responses of two target tissues to broaden the number of classes of chemical carcinogens that could be detected with either assay alone. The present experiments suggest that the SENCAR mouse does possess this advantage. This was most dramatically illustrated by the fact that DEN, generally found to be negative in the mouse skin initiation/promotion assay, illicited a positive response in the lung of the SENCAR under the same experimental conditions. A similar, but less effective argument can be made with respect to the fact that the tumorigenicity of FANFT was picked up in the mouse skin assay (confirming earlier studies in our laboratory) but did not produce a positive response in the lung of any strain. However, the low-level response in the skin to FANFT in the first experiment was not as apparent in the second experiment. Finally it must be noted that the advantage gained by using a single animal in these tests must be offset by the greater difficulty in scoring lung adenoma yields in strains other then the A/J. Within the context of the present study this difficulty seems unique to SENCAR mice.

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The research described in this paper has been peer and administratively reviewed by the United States Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does the mention of trade names or commercial products constitute endorsement or recommendation for use.

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Table 1. Results of combined lung adenoma and skin papilloms assays in SENCAR mouse following oral treatment with diethylnitrosamine

Chemical and dose[a]	% Animal with tumor	
	Lung adenoma	Papilloma
Diethylnitrosamine 50 mg/kg	85[b]	13
Emulphor	8	23

[a] Chemical was administered in single injection in emulphor to 60 animals. Two weeks later applications of TPA were made at 1.0 μg in 0.2 ml acetone 3 times weekly for 20 weeks to shaved backs of 40 animals. Results are reported as percent of animal having tumors after 40 weeks on study. Lung adenoma count include the total of 60 animals in the group whereas data on skin papillomas are derived only from the 40 animals receiving TPA promotion.

[b] Significantly different from control value ($p < 0.0001$) as determined by chi-square.

Table 2. Comparison of strain sensitivities to lung adenoma and skin papilloma induction in a combined protocol following interperitoneal treatment with known animal carcinogens[a]

			Exp. 2	Exp. 3			
Tumor	Chemical	Dose (mg/kg)	Sencar	Sencar	BALB/c	A/J	ICR
Lung adenoma	Ethyl Carbamate	300	1.38[b,c]	1.53[c]	0.14	4.92[c]	1.26[c]
	Benzo(a)pyrene	50[d]	0.37	0.49[e]	0.02	0.56[e]	0.16
	FANFT	500	0.07	0.07	0.11	0.08	0.09
	Acrylamide	50	0.38	0.42[e]	0.05	0.43	0.10
	Emulphor		0.29	0.14	0.03	0.16	0.05
	Water		0.22	0.04	0.03	0.11	0.00
Skin papilloma	Ethyl carbamate	300	1.68[c]	0.75[f]	0.08	0	0.68
	Benzo(a)pyrene	50[d]	0.70[c]	0.23	0	0	0.03
	FANFT	500	0.43	0.15	0	0	0.20
	Acrylamide	50	0.25[f]	0.38[c]	0	0	0.25
	Emulphor		0.18	0.08	0.05	0	0.08
	Water		0.08	0.05	0	0	0.13

[a] The indicated carcinogens were administered in a single I.P. injection in an emulphor or water (acrylamide) vehicle to 60 animals. Two weeks later applications of TPA were started. TPA doses varied for different strains and were applied in 0.2 ml acetone 3 times weekly for 20 weeks to the shaved backs of 40 animals in each group. SENCAR received 1.0 µg, BALB/c 5.0 µg, and both A/J and ICR-Swiss 2.5 µg of TPA per topical application 3 times weekly for 20 weeks.

[b] Values represent average number of tumors per animal.

[c] Significantly different from control value ($p < 0.01$) as determined by Fisher's Exact Test (Lung Adenoma) and the Gehan and Wilcox Test (Skin Papilloma)

[d] Benzo-(a)-Pyrene dose for experiment 2 was 100 mg/kg.

[e] Significantly different from control value ($p < 0.001$) as determined by Fisher's Exact Test (Lung Adenoma) and Gehan and Wilcoxon Test (Skin Papilloma).

[f] Significantly different from control value ($p < 0.05$) as determined by Fisher's Exact Test (Lung Adenoma) and the Cox Type Trend Test (Skin Papilloma).

PHORBOL MYRISTATE ACETATE AND CATECHOL AS SKIN COCARCINOGENS IN SENCAR MICE

by

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Key Words: SENCAR mice, phorbol myristate acetate, catechol, skin application, benzo(a)pyrene, beta-propiolactone, cocarcinogenesis.

ABSTRACT

The enhancement of the carcinogenicity of benzo(a)pyrene (BP) and of beta-propiolactone (BPL) by the mouse skin cocarcinogens phorbol myristate acetate (PMA) and catechol were examined in female SENCAR mice, thirty per group. The carcinogen and cocarcinogen were applied simultaneously, three times weekly for 490-560 days. BP and BPL were used at constant doses of 5 and 50 ug, respectively in all experiments. PMA was used at three doses, 2.5, 1.0 and 0.5 ug, per application and catechol was used at one dose, 2 mg per application. Control groups included animals which received carcinogen only, cocarcinogen only, acetone only and no treatment groups. The carcinogenicity of BP and BPL were enhanced by the cocarcinogens, particularly in terms of tumor multiplicity. For both carcinogens the most marked cocarcinogenic effects were observed at the lowest dose of PMA used, i.e. 0.5 ug per application; this applied for days to first tumor, animals with tumors, tumor multiplicity and incidence of malignant skin tumors. Catechol applied alone did not induce any tumors; with PMA alone there were significant incidences of benign and malignant tumors, e.g. at a dose of only 0.5 ug per application 15 of 30 animals had 28 tumors, 5 of which were squamous carcinomas. In two-stage carcinogenesis experiments with 7,12-dimethylbenz(a)anthracene as initiator and PMA as promoter SENCAR mice showed a greater susceptibility to tumor induction when compared to ICR/Ha mice used in earlier work. This

was most notable for rate of tumor appearance and tumor multiplicity.

Introduction

The two-stage model for chemical carcinogenesis was first described in the classic work of Berenblum (1,2) who used mouse skin as the route of exposure. At the same time Berenblum showed that remarkable enhancement of the carcinogenic activity of benzo(a)pyrene (BP) occurs by what was then called croton oil or its concentrate, croton resin, when the two agents were applied simultaneously and repeatedly on mouse skin. The latter type of experimental protocol was referred to by Berenblum as cocarcinogenesis (1). Since that time, the mouse skin test system has been used in many laboratories for studies on two-stage carcinogenesis and, to a lesser extent, cocarcinogenesis and these endeavours have been reviewed (3,4). The two protocols as used by Berenblum are shown in Table 1.

TABLE 1

(see page 410)

One result of the early work was the development of mouse lines that would be particularly sensitive to tumor induction in two-stage carcinogenesis. This was first accomplished in the pioneering work of Boutwell (5) who explored the development of skin tumor susceptible (STS) mice by careful inbreeding experiments using two-stage carcinogenesis for selection of STS mice. The necessary requirements of inbreeding and the small sizes of colonies used for the development of STS mice resulted in the decline of reproductive performance in these mice. This was alleviated by cross-breeding male STS with Charles River female CD-1 mice. The details and history of the development of the susceptible mice thus developed was recently described (6). These mice, then maintained at the Oak Ridge National Laboratory, have since then been called SENCAR mice.

In the course of work on carcinogens, tumor promoters and cocarcinogens in tobacco smoke condensate it became necessary to examine some major components of tobacco smoke for their tumor promoting and/or cocarcinogenic properties. Many of the tobacco phenols, which were suspect as tumor promoters based on the finding that phenol itself is a weak tumor promoter (7), proved to be inactive as tumor promoters using the two-stage protocol (8). In the human exposure to cigarette smoke there is simultaneous and repeated intake of aromatic hydrocarbon carcinogens and many other chemicals, some of which were considered potential cocarcinogens. This proved to be the case for such compounds as catechol, pyrene, fluoranthene, undecane and several others in a series of structurally unrelated chemicals which were inactive as tumor promoters in two-stage carcinogenesis and also were not carcinogens (8,9).

For continued studies on environmentally important factors, particularly chlorinated humic acids which occur in drinking water, it became relevant to explore the use of SENCAR mice because of their reported increased susceptibility to skin tumor induction (6).

In the chemical and biological work which resulted in the isolation and structure elucidation of the phorbol esters from croton oil, random-bred ICR/Ha mice, were used (10). These mice were also used in our subsequent work on the promoter phorbol myristate acetate (PMA, also referred to as TPA) including dose response studies, the effect of aging in two-stage carcinogenesis and other studies (4). The same mouse strain was also used in our earlier studies on mouse skin cocarcinogens (9). Hence, it became relevant to compare our earlier results using ICR/Ha mice with SENCAR mice.

When the work described in the present report was undertaken in early 1980 there were reports which indicated that SENCAR mice are more susceptible to mouse skin tumor induction than CD-1 mice (6) and BALB/c mice (11) in two-stage carcinogenesis using several endpoints of bioassays. In subsequent work, DBA/2 mice were found to be as sensitive as SENCAR mice, also in two-stage carcinogenesis with PMA as promoter (12). The sensitivity of various mouse strains, including SENCAR, for complete carcinogenesis and for the initiation-promotion protocol has been rank-ordered (13).

The work described in this report deals with the use of SENCAR mice in skin tumor induction using mainly cocarcinogenesis protocols with BP and beta-propiolactone (BPL) as carcinogens together with the mouse skin cocarcinogens PMA and catechol. The findings obtained with SENCAR mice, and described here, are compared with the earlier studies in ICR/Ha mice (4,9,14).

Methods

Animals, housing and treatment. - Female SENCAR mice were obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn., at ~4 weeks of age and were put on test at age ~6 weeks. There were thirty animals per group. All chronic experiments were preceded by short-term evaluations lasting 4 to 6 weeks in order to determine acceptable doses for chronic exposure. The doses selected for short-term evaluation were based on those used earlier with the same chemicals in ICR/Ha mice (9,15). For the two-stage experiments, the test duration was 550 days and for cocarcinogenesis, the test duration was 490 or 560 days. The dorsal skins of all mice were shaved at the beginning of experiments and then as needed during the tests. Animal housing and care was the same as that used before (9). All animals were weighed at bimonthly intervals and records of weight gains were kept. Animals were observed for tumor appearance daily, scored and observations charted once monthly. All solutions were applied to the dorsal skin in 0.1 ml acetone by micropipette except for initiation with DMBA by subcutaneous injection; DMBA was injected in the left flank in 0.05 ml tricaprylin per injection. The appropriate vehicle and no-treatment control groups were included; these and doses of chemicals used are presented under

Results. In two-stage carcinogenesis experiments, a single dose of DMBA was followed two weeks later by thrice weekly application of PMA. In the cocarcinogenesis experiments, the two agents (BP or BPL and cocarcinogens PMA or catechol) were applied simultaneously for the duration of the test. Animals were killed at the conclusion of tests or when in moribund condition, and necropsied . Skin lesions were excised, sectioned and submitted for histopathologic diagnosis. Lesions were counted as papillomas when they reached a size of 1 mm^3 and persisted for more than 30 days. Lesions which regressed by 30 days or earlier were not counted in the cumulative total tumors.

Chemicals. DMBA (Eastman Organic Chemicals, Rochester, N.Y.) was purified by recrystallization from 95% ethyl alcohol to constant m.p., 122.5-123°; fresh solutions were prepared immediately before use in bioassays. BP (Aldrich Chemical Company) was recrystallized from benzene:methanol (1:3) to constant m.p., 174-176°. BP solutions were prepared once per month from freshly recrystallized material. BPL (Sigma Chemical Company, St. Louis, Mo.) was >99% pure by gas chromatography and used as such. Solutions of BPL were prepared freshly once per week. Catechol (Fisher Scientific Co., Fairlawn, N.J.) was purified by recrystallization from toluene, m.p. 100-103°. Its purity was checked by thin-layer chromatography on silica gel (chloroform:ethyl acetate, 1:1, R_F 0.68). All solutions were prepared in anhydrous acetone and all pure chemicals and their solutions were stored in amber-colored containers at 4°. PMA used in the two-stage experiments was prepared from croton oil, purified and its purity checked by thin-layer chromatography as described before (15). PMA for the cocarcinogenesis experiments was obtained from Lifesystems Co. (Newton, MA) and from CCR Inc. (Eden Prairie, MN). Its purity was checked by thin-layer chromatography as before (15).Solutions for cocarcinogenesis bioassays were prepared by combining proper concentrations of carcinogens and cocarcinogens once weekly so that the final concentrations in the bioassay test solutions were those shown under Results. They were stored at 4° in amber-colored containers. Solutions of BPL combined with catechol were stored at -20°. In order to insure that no reaction occurred between BPL and catechol, bioassay solutions stored under these conditions were checked for purity by gas chromatography six days after preparation. Only the peaks for the pure components were noticed and traces of possible reaction products or other impurities were not detectable.

Results

The sensitivity of SENCAR mice to two-stage carcinogenesis on skin using protocols that were used in earlier work (4,14,15), was examined with DMBA as initiator at two doses (0.5 ug and 0.1 ug) by the subcutaneous route and at 5 ug by the topical route. PMA was applied topically as promoter at 0.5 ug/application throughout. The low dose of PMA used, 0.5 ug, per application was based on short-term evaluations of SENCAR mice for skin irritancy. The results of this experiment are shown in Table 2.

Table 2
(see page 411)

Topical application of DMBA followed by PMA gave the expected high incidence of tumors, benign and malignant, in the DMBA-PMA group and also showed that DMBA and PMA by themselves induced benign and malignant tumors. The latter tumor incidences were markedly higher than that observed earlier (4,14,15) in comparable control groups with ICR/Ha mice.

DMBA administered subcutaneously at one-tenth and one-fiftieth of the dose used for skin initiation was still effective as initiator but the tumor incidences were markedly lower than by skin application.

In the second series of experiments, the carcinogens BP or BPL were applied simultaneously and repeatedly with the mouse skin cocarcinogens PMA or catechol. In these experiments, PMA was used at three dose levels with constant dose of carcinogen. Catechol was used at one dose only. The results of these experiments together with the results from app[illegible]e control groups are shown in Table 3. With BP or BPL as carcinogens higher incidences of tumors were induced with decreasing doses of

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Table 3
(see page 412)

PMA, from 2.5 to 0.5 ug/application. Days to first tumors ranged from 49-85 days. The rates of tumor appearance reflected clearly the inverse relationship between dose of PMA and total numbers of tumors as shown figures 1 and 2.

Figures 1 and 2
(see pages 414 and 415)

The control group, BP or BPL alone resulted in late appearance of first tumors and lower incidences of tumors after 500 days on test than were seen with simultaneous application of PMA or catechol.

Discussion

In the evaluation of the relative susceptibility of two or more strains of mice to skin tumor induction by chemicals, it is necessary to consider: (a) treatment regimen, which can be a single chemical applied once only or repeatedly (carcinogenesis), single treatment with an initiator followed by repeated application of a promoter (two-stage carcinogenesis) or repeated treatment with two chemicals applied simultaneously (cocarcinogenesis); (b) dose-response information; (c) class of chemical used, e.g., direct-acting or indirect-acting carcinogen, type of promoter or cocarcinogen; (d) duration of treatment and subsequent observation; (e) animal survival; (f) incidence of spontaneous tumors; and (g) experimental observations which includes: time to first tumor, rate of tumor appearance, tumor multiplicity, tumor regression, and incidence of malignant tumors. Because of the differences in goals of various investigators, it is rarely possible to obtain all the desired information.

In the present work the main purpose was to examine the susceptibility of SENCAR mice to tumor induction in two-stage and cocarcinogenesis experiments and to compare these findings with the same chemicals used in our earlier work in the same experiments with ICR/Ha mice. Only one two-stage carcinogenesis experiment was carried out in this work, Table 2, which is comparable to an earlier experiment (15). In both experiments the dose regimen was one application of DMBA, 5 ug, followed by PMA, 0.5 ug, thrice weekly for the duration of the tests. In ICR/Ha mice 13 of 20 animals bore tumors after 365 days on test of which three bore squamous carcinoma of the skin (15). In SENCAR's all 30 bore tumors, 23 of which had squamous carcinomas and two sarcomas. The test duration was 365 days for ICR/Ha and 400 days for SENCAR mice and hence the tests were comparable. There was little difference in days to first tumors which was 36 for ICR/Ha and 44 for SENCAR. The most marked difference was tumor multiplicity which averaged 2.7 per mouse for ICR/Ha and 23.6 for SENCAR. In addition, the rate of tumor appearance was much more rapid in SENCAR's compared to ICR/Ha, which is similar to what has been observed in comparing SENCAR's to other mouse strains (13).

The differences in tumor response between the two strains depends on the dose of PMA used as promoter. SENCAR mice are more sensitive to the irritant effect of PMA than ICR/Ha and the optimal dose of PMA for SENCAR mice was 0.5 ug per application (Table 2) and for ICR/Ha it was 25 ug (15) in terms of mice with tumors, tumor multiplicity and rate of tumor appearance. This comparison is based on a dose of 5 ug of DMBA as initiator, a 14 day interval between initiator and promotion and three times weekly application of PMA. It is not possible to extrapolate from this type of experiment to other tumor initiators or promoters or to mouse skin assays for complete carcinogens.

In another comparative two-stage carcinogenesis experiment, BPL was used as the initiator, 30 mg, once only in 0.1 ml acetone administered topically, followed by PMA, 5 ug per application, three times weekly for 320 days using a three day interval between initiation and promotion (14). There were 20 female ICR/Ha and SENCAR mice per group. The rate of tumor appearance in this experiment is shown in Figure 3.

Figure 3
(see page 416)

Unlike many other comparative studies which showed SENCAR mice to be the more sensitive strain (13), ICR/Ha mice resulted in more rapid tumor appearance and final tumor yield. Days to first tumor were close to 35 days for both groups. The final tumor yields were 16 SENCAR mice with 55 tumors (three carcinomas) and

18 ICR/Ha mice with 100 tumors (two carcinomas, one sarcoma).

The two differences between this experiment and the one shown in Table 2 are: (a) the use of an indirect-acting carcinogen, DMBA, in one case and a direct-acting carcinogen, BPL in the other; and (b) the use of a higher dose, 5 ug, of PMA with BPL initiation compared to 0.5 ug with DMBA initiation. It is likely that the high dose of PMA resulted in decreased tumor yields in SENCAR mice as was observed in the cocarcinogenesis experiments summarized in Table 3.

It has been observed in studies with PMA as promoter in two-stage carcinogenesis experiments that control groups receiving PMA alone develop a low incidence of benign and malignant skin tumors (16). In the present study using only SENCAR mice,the induction of benign and malignant skin tumors with PMA alone at three dose levels, shown in Table 3, was considerably higher than that observed with PMA in ICR/Ha mice (15). The lowest dose used, 0.5 ug PMA, thrice weekly, induced 28 tumors, in 15 animals, five of which had carcinomas (Table 3). Similar findings were made when the effect of PMA alone on SENCAR mice were compared with other mouse strains, CD-1 mice (6) and BALB/c mice (11) both of which were found to result in lower tumor incidences than SENCAR mice.

The importance of dose response studies is illustrated in Figures 1 and 2 in which the results of BP or BPL applied simultaneously with PMA (cocarcinogenesis) at three dose levels are compared. In both cases, the rate of tumor appearance and highest tumor yields were obtained at the lowest dose, 0.5 ug, of PMA used. This is probably due to increased skin inflammation and hyperplasia as the dose of PMA is increased. During the course of the tests listed in Table 3, the skin-damaging effects of 2.5 ug of PMA applied thrice weekly became apparent and this required, and was remedied by, decreasing the frequency of application to twice weekly as noted in Table 3.

In the present work studies on mouse skin cocarcinogenesis were extended by examining the phenomenon in SENCAR mice, by including in the present study the direct-acting carcinogen BPL and by including the use of several doses of PMA as cocarcinogen. The results of the latter set of experiments emphasized the importance of using several doses of PMA in these cocarcinogenesis experiments, as was done in earlier studies on dose-response in two-stage carcinogenesis with PMA as promoter (15). The potency of PMA as a skin irritant necessitates careful consideration of this factor in comparing different mouse strains in skin carcinogenesis by the two protocols given in Table 1.

In addition to PMA the cocarcinogen catechol was used in the present work. The high dose of catechol was arrived at on the basis of early short-term evaluations of its skin irritancy (9). Since it did not show any notable skin irritancy at 2 mg per application, this dose was selected for use in cocarcinogenesis experiments. No dose response experiments were carried out with this chemical which is remarkably simple in chemical structure, yet biologically active as cocarcinogen. Catechol is completely devoid of tumor-promoting or carcinogenic activity in mouse skin using both ICR/Ha (9) and SENCAR mice (present study). Further work with this compound is indicated, both in terms of biological activity, particularly dose response, and mode of action.

From the work described in this report and findings reported earlier in mouse skin cocarcinogenesis experiments from the same laboratory with ICR/Ha mice (9,14), limited comparative conclusions were made as listed in Table 4. These conclusions apply only to the experiments performed in our laboratory and within the dose ranges used, duration of treatment and for the chemicals used. Extrapolation to other experiments and experimental conditions as enumerated at the beginning of this Discussion were not attempted.

Table 4

(see page 417)

ACKNOWLEDGMENTS

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Table 1. Protocols for Two-stage Carcinogenesis and Cocarcinogenesis

Two-stage: Single application of initiator (DMBA, BP or BPL) followed by repeated applications of promoter (PMA), i.e. sequential application of chemicals.

Cocarcinogenesis: Repeated and simultaneous application of carcinogen (BP or BPL; low dose) and cocarcinogen (PMA or catechol).

Table 2. Two-stage Carcinogenesis in SENCAR mice. Initiation By Subcutaneous Injection and By Topical Application of DMBA. PMA By Topical Application. Test Duration 550 Days. Median Surivival Time > 550 days; 30 ♀ Mice Per Group.

Treatment and dose (µg)	Days to first tumor	Number of mice with tumors / Total tumors	Number of mice with malignant tumors[a]
Subcutaneous[b]:			
DMBA, 0.5, PMA 0.5	93	14/22	5 Ca, 1 Sa
DMBA, 0.1, PMA, 0.5	120	16/32	10 Ca
DMBA, 0.5, acetone, 0.1 ml	540	1/1	1 Ca
DMBA, 0.1, acetone, 0.1 ml	-	0	0
Topical[c]			
DMBA, 5, PMA, 0.5[d]	44	30/692	23 Ca, 2 Sa
DMBA, 5, acetone, 0.1 ml	140	4/8	3 Ca
PMA, 0.5	170	13/22	4 Ca, 1 Sa
Acetone, 0.1 ml	374	2/2	0
No treatment[e]	513	1/1	0

[a] Ca: squamous carcinoma; Sa: sarcoma.

[b] Injected subcutaneously in 0.05 ml tricaprylin in left flank once only followed 14 days later by three times weekly application of PMA in 0.1 ml acetone to dorsal skin.

[c] Single dorsal skin application of DMBA in 0.1 ml acetone followed 14 days later by three times weekly application of PMA in 0.1 ml acetone.

[d] Test duration: 400 days, median survival time: 311 days.

[e] Sixty mice.

Table 3. Cocarcinogenesis in SENCAR Mice. 30 ♀ Mice Per Group

Test Duration days	Median Survival time, days	Day to first tumor	Mice with tumors / Total tumors	Mice with malignant tumors[c]
560	372	50	27/334	21 Ca, 1 Sa
490	344	49	27/423	16 Ca
490	322	49	29/473	22 Ca
560	310	179	30/192	25 Ca
560	447	69	16/36	10 Ca, 1 Sa
490	437	85	24/99	11 Ca, 2 Sa
490	404	57	28/124	19 Ca
560	>560	153	14/43	9 Ca
560	555	295	24/79	19 Ca
560	>560	293	2/4	1 Ca
560	515	277	10/12	4 Ca
490	>490	141	15/24	8 Ca
490	>490	85	15/28	5 Ca
560	>560	-	0	0
500	>500	-	0	0
520	>520	-	0	0
570	>570	-	0	0

Footnotes to Table 3.

[a] Carcinogens: BP and BPL; cocarcinogens:PMA and catechol applied to the dorsal skin simultaneously in 0.1 acetone three times weekly for the duration of the test except where noted. For preparation of solutions, see text, Methods.

[b] Treated three times weekly for 120 days, then two times weekly for 440 days due to severe irritancy of PMA at dose used.

[c] Abbreviations: Ca - squamous cell carcinoma; Sa - sarcoma.

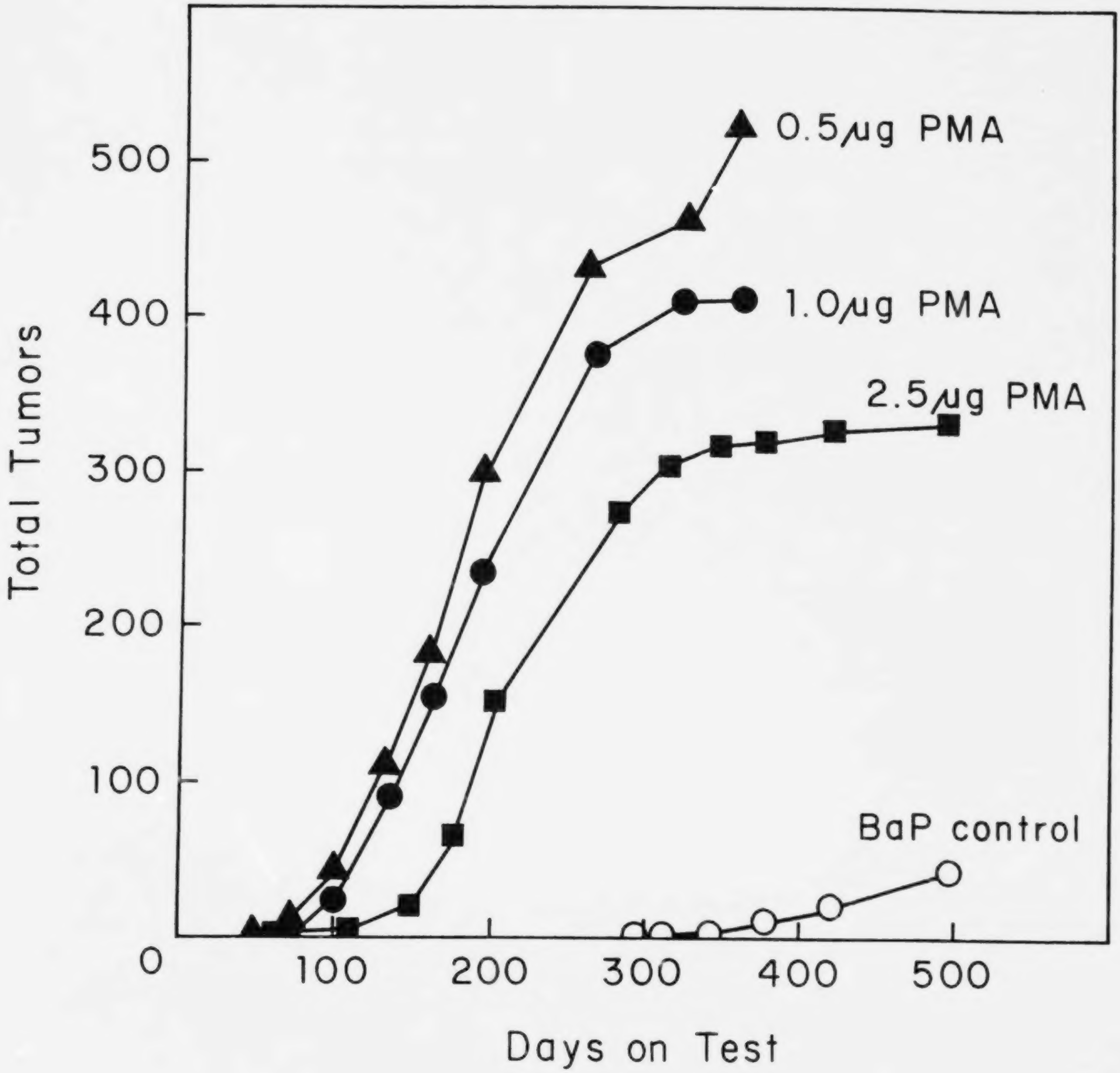

FIGURE 1 Rate of tumor appearance in skin cocarcinogenesis with 30 female SENCAR mice per group; BP, 5.0 ug, PMA at doses indicated. Applied simultaneously three times weekly.

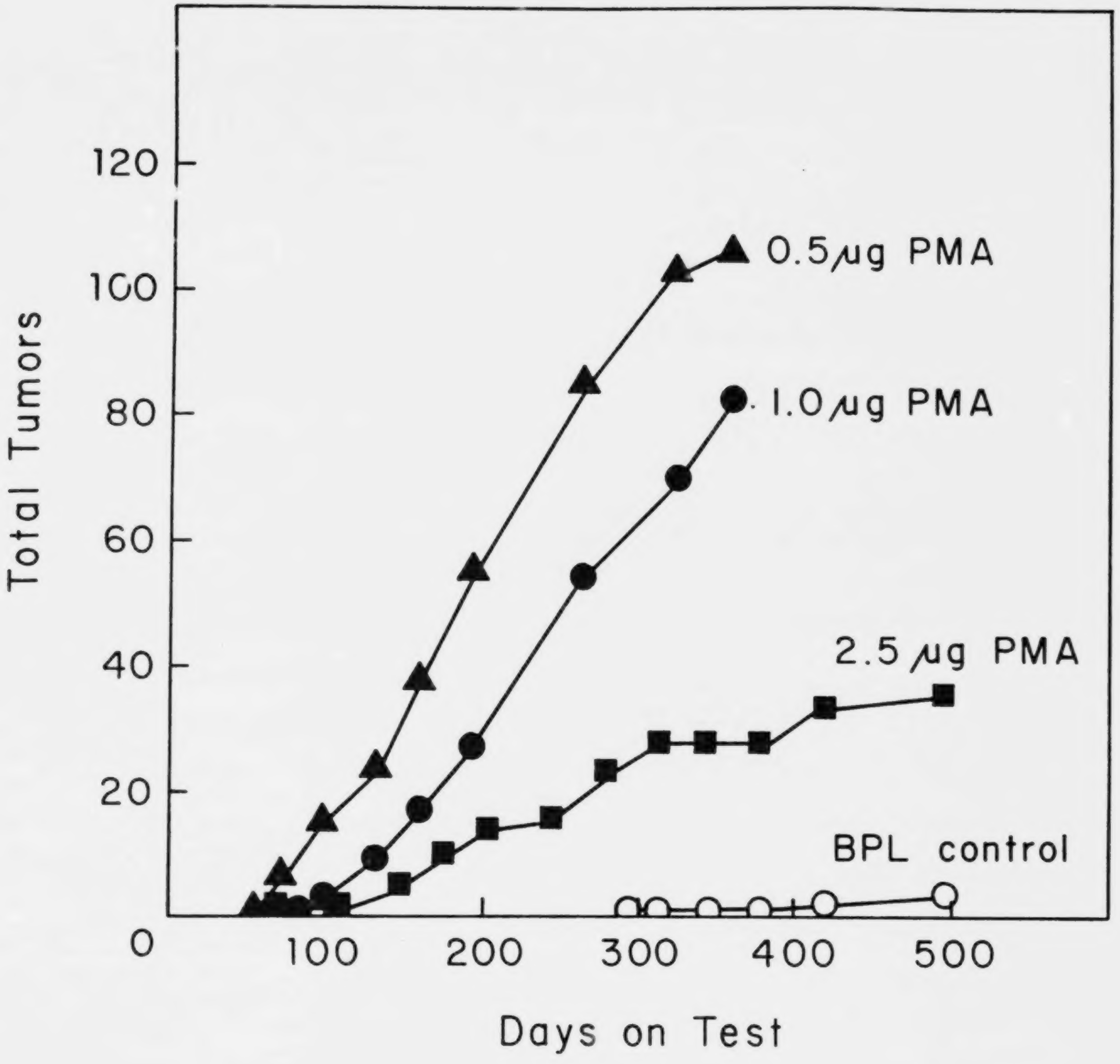

FIGURE 2. Rate of tumor appearance in skin cocarcinogenesis with 30 female SENCAR mice per group; BPL, 50 ug, PMA at doses indicated. Applied simultaneously three times weekly.

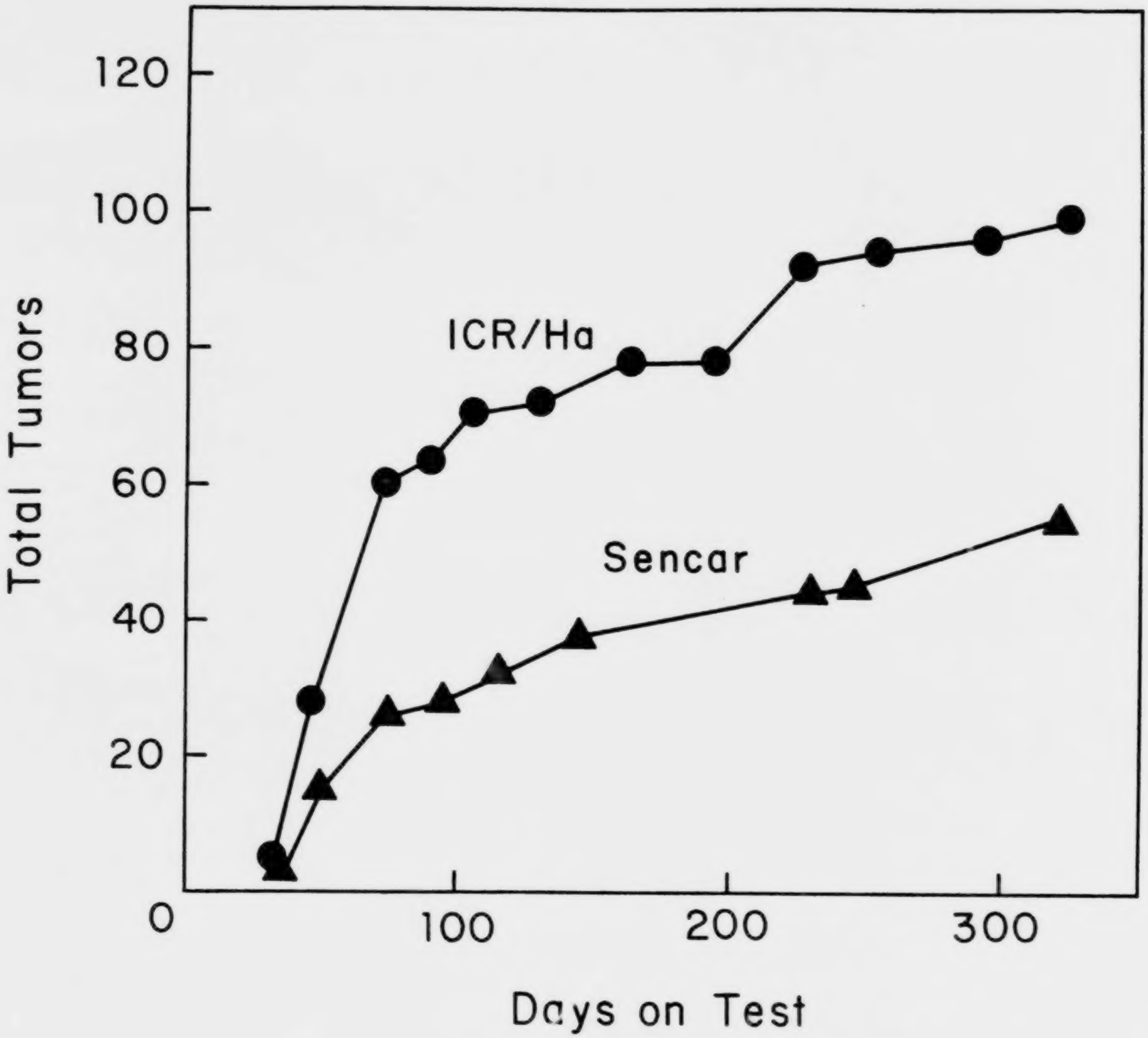

FIGURE 3 Rate of tumor appearance in two-stage carcinogenesis with ICR/Ha and SENCAR mice, 20 females per group; 30 mg BPL in 0.1 ml acetone once only, followed three days later by 5 ug PMA in 0.1 ml acetone, three times weekly for 320 days.

Table 4. Conclusions. Skin Tumorigenesis In Female ICR/Ha And SENCAR Mice

Sencar > ICR/Ha

1. Tumor-multiplicity.
2. Earlier peak response.
3. Both 1 and 2 occur with polycyclic aromatic hydrocarbons and PMA.

Sencar = ICR/Ha

1. Minimum latent period.
2. Pattern of morphologic development of skin tumors by two stage and cocarcinogenesis protocols.
3. Incidence of squamous carcinoma of the skin in lifetime tests.

ICR/Ha > Sencar

1. Tumor induction in two-stage carcinogenesis after BPL initiation and promotion by PMA.

SPONTANEOUS TUMORS IN SENCAR MICE

by

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Key Words: SENCAR mice; spontaneous tumors; carcinogenicity testing; animal models.

ABSTRACT

A total of 305 female and 36 male SENCAR mice in six different groups were observed for 540 to 875 days for tumors as well as non-neoplastic lesions. Three of these groups were observed for their lifespans. One hundred and twenty three females and 36 males, observed for their lifespans, 800 to 875 days, showed median survival times ranging from 730 to 745 days. The average peak body weights were 42.4 grams for females and 50.0 grams for males. In the animals observed for their lifespans, the total tumor incidence was 65.6% for females and 69.4% for males. Both sexes showed a high incidence of papillary tumors of the lung, 16-39%, which was independent of aging. Females showed a 5.1 to 21.0% incidence of mammary tumors which increased with aging. Males showed a high incidence of hyperplastic nodules of the liver, i.e. 12 of 36 animals. Other frequently occurring tumors were leukemia and papillomas of the forestomach.

Introduction

In a companion report from this laboratory (1), we described the use of female SENCAR mice in the bioassay of chemicals for cocarcinogenic and tumor-promoting activity and, in some instances, compared the skin tumor responses in these animals with that observed in our earlier work in which female ICR/Ha mice were used (2).

During the course of the above work, and including earlier trial experiments with SENCAR mice (3), we have accumulated some background information on the incidence of spontaneous tumors (benign and malignant) and non-neoplastic lesions in SENCAR's. In these no-treatment control groups 305 females and 36 males were observed in six different experiments for periods ranging from 540 to 875 days.

Because of the current interest in the use of these mice for skin carcinogenesis assays, which was the main topic of the conference at which the above paper (1) was presented, it was considered useful to present our findings on the incidence of spontaneous lesions in SENCAR mice.

The findings reported here were accumulated in the course of mouse skin cocarcinogenesis research. Hence, we do not have the detailed results which would have been produced from a protocol designed for the purpose of studying spontaneous tumors in SENCAR mice.

Methods

All mice were obtained from Oak Ridge National Laboratories, Oak Ridge, TN. They were used as no-treatment control groups for concurrently running skin cocarcinogenesis and two-stage experiments, some of which have been reported earlier (1,3). The six groups of mice represent animals used over a period of seven years in this laboratory. They were four to seven weeks of age at time of arrival in the laboratory. Animals were allocated randomly and numbered by toe-clipping during the week of arrival. After one week, they were assigned to experimental groups as no-treatment controls. Mice were housed, five per stainless steel cage on heat-treated hardwood chip bedding. Males and femals were housed in separate animal rooms and males showing aggressive behavior were separated out and housed individually. Rooms were maintained at 68-72°F and 50 per cent relative humidity. The fluorescent lighting used was on a twelve hour on-off cycle and fresh air was supplied at the rate of 12-15 changes per hour. The animals had free access to Purina Rodent Chow (number 5001) and tap water. The six groups and relevant information are given in Table 1.

Table 1
(see page 432)

Groups 1 to 3 were matched with skin application groups and hence were killed at the termination of the experiment. Groups 4 to 6 were kept to end of their lifespans. Animals in moribund condition were killed. Necropsies did not include the cranium and routine sections of tissues were not taken for groups 1 to 3. Only those tissues which appeared abnormal by gross examination were taken for histopathology. In groups 4-6, routine sections of the following clinically normal tissues were taken in addition to abnormal tissues: lung, liver, kidney, stomach, spleen and skin. Tissues were fixed in formalin and stained with hematoxylin and eosin; special stains were not used.

Results

Survival. - The survival curves for group 4,5 and 6, which were allowed to live out their lifespans are shown in Figure 1.

Figure 1
(see page 433)

The median survival times for these three groups are shown in Table 1.

Body weight. - Body weights of the six groups of animals are shown in Figure 2.

Figure 2
(see page 434)

The average peak body weight was 42.4 grams for females and peak body weight for the one group of males was 50.0 grams.

Tumors. - In Table 2 all tumors are listed as number of mice with tumor and in percent for purposes of comparison. The mammary tumors were defined by the following criteria: when proliferation of both the epithelium and stroma were present, the tumor was classified as a fibroadenoma; tumors designated adenocarcinoma also showed signs of necrosis. When neither of the above were present, we used the generic term mammary tumor and these are designated unclassified tumors in Table 2.

Table 2

(see page 435)

Non-neoplastic lesions. - Non-neoplastic lesions were seen in all six groups of SENCAR mice examined and are listed in Table 3.

Table 3

(see page 438)

With the few exceptions noted below, these lesions were all clinically apparent at necropsy and thus represent a conservative number. In the males, group 5, cystic dilatation of the seminal vesicles was probably an age-related lesion. The changes seen in the male skin and some of the inflammatory changes seen in the kidneys may have been attributable to amyloidosis. We did not use special stains for amyloid since non-neoplastic lesions were not of prime interest in our study.

Routine sections. - The numbers of routine sections of various organs taken from groups 4-6 and the diagnoses are shown in Table 4.

Table 4
(see page 441)

The total number of clinically normal sections from all tissues listed in Table 4 was 143. Of these only 10, i.e. about 7% were diagnosed as tumors or non-neoplastic lesions. Thus, these routine sections did not markedly alter the overall tumor and non-neoplastic lesion incidences shown in Tables 2 and 3.

Discussion

An examination of the tumor incidences in SENCAR mice used in this laboratory, listed in Table 2, revealed that five tumor types occur most frequently. These tumor types and the frequency of their occurrence are summarized in Table 5.

Table 5
(see page 442)

These tumors occurred in one or more of the six groups with a frequency of above 10%. With the exception of group 3, the overall tumor incidences were greater than 50%. The most commonly occurring lung tumors were benign papillary tumors and this ranged between 16 and 39% in the six groups. The incidence of lung tumors does not appear to be age-related, e.g. group 1, which was observed for 540 days had essentially the same lung tumor incidence as groups 5 and 6 which were observed for 815 and

875 days, respectively. In another study (4) the most frequently observed tumors were those of the lung in CD-1 mice of both sexes.

Male mice showed a high incidence of hyperplastic nodules of the liver (group 5), 33.3 % compared to females which showed a range of 0-2.4%. This has also been observed in B6C3F1 hybrids and other mouse strains (5).

The mammary tumor incidences summarized in Table 5 showed a distinct increase with aging of the animals from 5.1% in group 1 (540 days) to 22% in group 6 (875 days).

It was of interest to compare median survival times and body weights of SENCAR mice from some of the control groups discussed here with ICR/Ha mice used in most of our earlier studies on promoters, cocarcinogens and whole carcinogens. Groups 5 and 6 from the present report were compared with ICR/Ha mice of both sexes used as control groups in an earlier study (6). This comparison is illustrated in figure 3. The markedly higher weight and increased median survival times of SENCAR's compared to ICR/Ha of both sexes are noteworthy.

Figure 3

(see page 443)

In the course of the preparation of this report and another one for this symposium (1), the differences in results obtained in different laboratories with the same strain of mice became obvious. This has again stressed to us the importance of standardization of laboratory animal care and handling procedures. So many variables, often overlooked as of minor revelance, are in fact important for the comparison of chronic animal bioassay results amongst different laboratories. The importance of all of these variables has been discussed and reviewed (7).

From the papers presented at this symposium, it was clear that the SENCAR mouse will be valuable for mouse skin tumorigenesis studies particularly in two-stage, i.e. initiation-promotion experiments. In this regard the utility of SENCAR mice for carcinogenesis studies relating to tissues and organs other than skin merits further attention. This is important because *in vitro* models, while most valuable, must continue to be regarded as adjuncts to, rather than replacements for *in vivo* animal bioassays. The latter view is shared by other researchers in toxicology (8,9).

Our findings from the present work and from that presented elsewhere in this volume (1) indicate the need for further work on the utility of the SENCAR mouse in toxicology research.

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Table 1. Groups of Mice Observed For Spontaneous Lesions

Group number and sex	Number of mice		Median survival time (days)	Duration of observation (days)
	At start	Necropsied		
1, ♀	60	59	>540	540
2, ♀	65	63	>600	600
3, ♀	57	56	>605	605
4, ♀	80	78	745	800
5, ♂	36	36	750	815
6, ♀	43	41	730	875

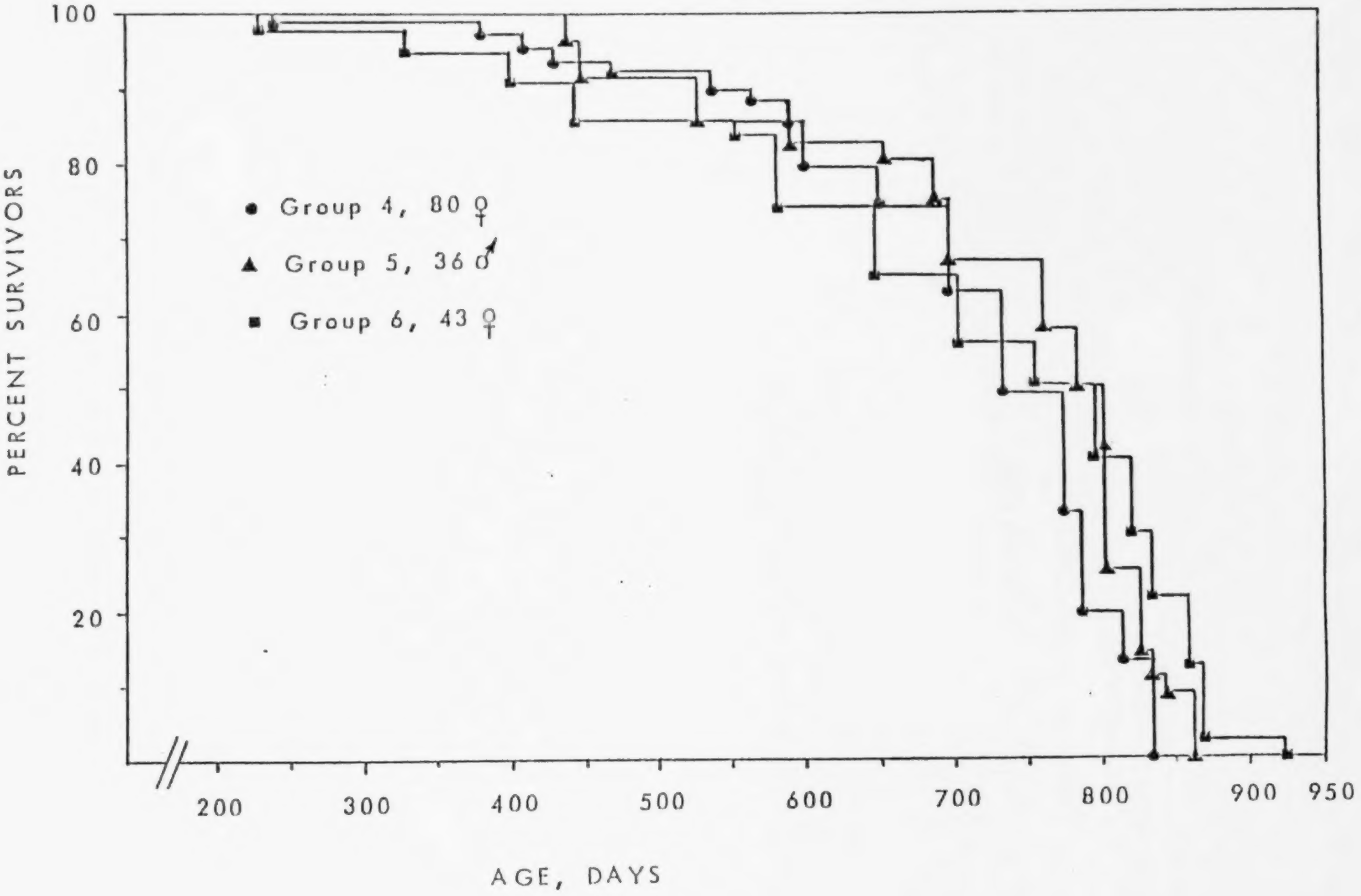

FIGURE 1. Survival curves for groups 4-6 of SENCAR mice.

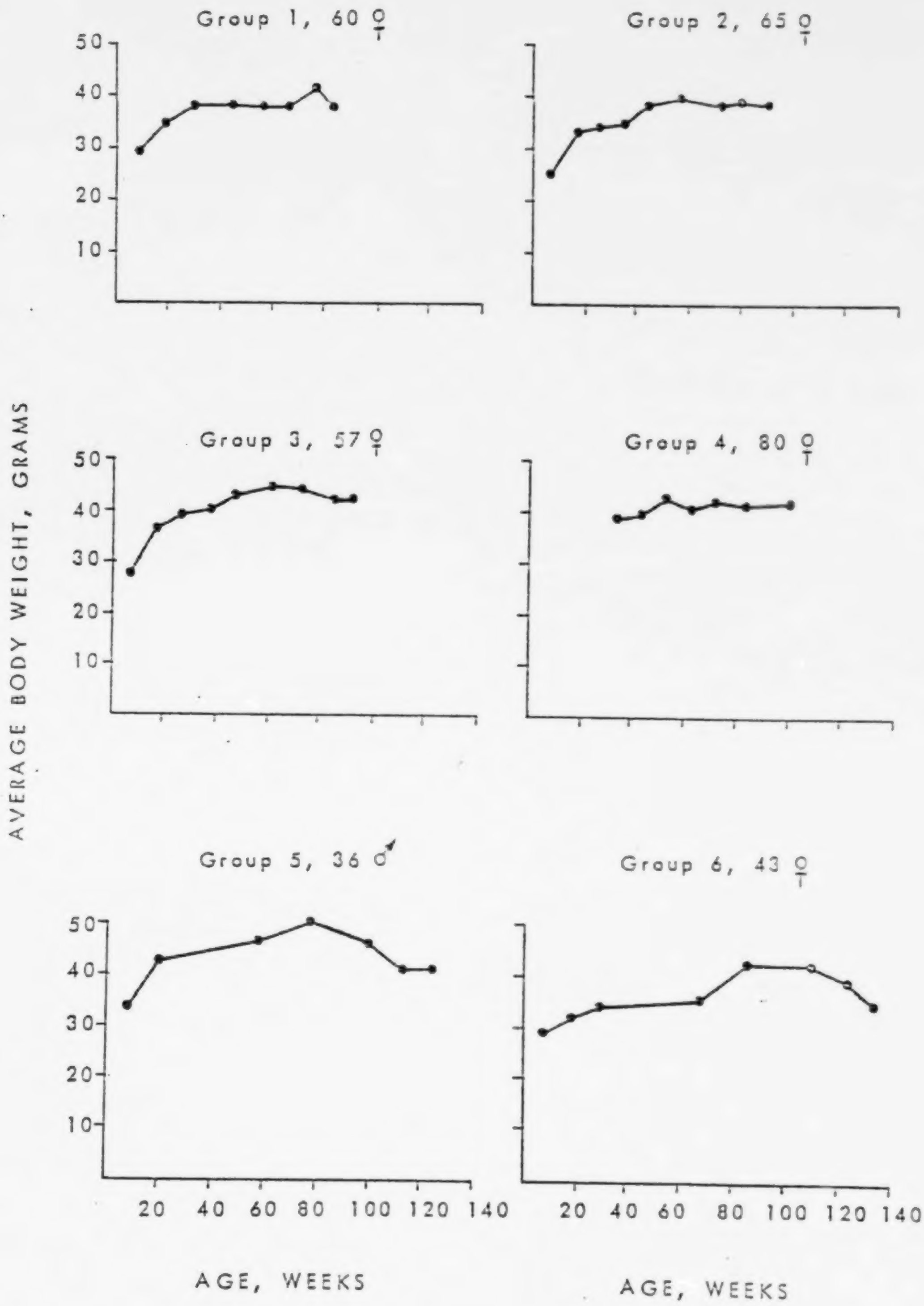

FIGURE 2. Average body weights of groups 1-6 of SENCAR mice.

Table 2. Tumor Incidences In SENCAR Mice

		Number of mice with tumors (per cent)					
Site	Tumor type / Group no.:	1	2	3	4	5	6
Lung	Papillary tumor	19 (32.2)	18 (28.6)	9 (16.1)	20 (25.6)	12 (33.3)	15 (39.0)
	Papillary tumor and secondary carcinoma						1 (2.4)
	Secondary mammary carcinoma				1 (1.3)		
Liver	Hyperplastic nodule	1 (1.7)	1 (1.6)			12 (33.3)	1 (2.4)
	Hemangioma				1 (1.3)	4 (11.1)	1 (2.4)
Mammary gland	Unclassified	3 (5.1)	8 (12.7)	6 (10.7)	16 (20.5)		6 (14.6)
	Fibroadenoma		1 (1.6)				
	Carcinoma			1 (1.8)	2 (2.6)		2 (4.9)
	Adenocarcinoma			1 (1.8)			
	Squamous cell carcinoma						1 (2.4)
Forestomach	Papilloma	5 (8.5)	4 (6.3)	3 (5.4)	5 (6.4)	6 (16.7)	6 (14.6)
Glandular stomach	Undifferentiated malignant tumor						1 (2.4)
Duodenum	Adenoma					1 (2.8)	
Kidney	Carcinoma				1 (1.3)		

Table 2. Tumor Incidences in SENCAR Mice (continued)

		Number of mice with tumors (per cent)					
Site	Tumor type	Group no.: 1	2	3	4	5	6
Spleen	Hemangioma						2 (4.9)
Skin	Papilloma			1 (1.8)		1 (2.8)	
	Sarcoma					1 (2.8)	1 (2.4)
Uterus	Leiomyoma			2 (3.6)			
	Sarcoma				1 (1.3)		
Ovary	Adenoma				1 (1.3)		
	Carcinoma			1 (1.8)	1 (1.3)		
	Brenner tumor				2 (2.6)		
	Hemangioma						1 (2.4)
	Interstitial cell tumor						1 (2.4)
	Benign epithelial tumor						1 (2.4)
Adrenal gland	Secondary carcinoma						1 (2.4)
Bone	Sarcoma				1 (1.3)		
Blood vessel	Hemangioma		1 (1.6)				
Ear duct gland	Squamous cell carcinoma		1 (1.6)				
Paraspinal cord	Squamous cell carcinoma (undetermined origin)		1 (1.6)				

Table 2. Tumor Incidences in SENCAR Mice (continued)

			Number of mice with tumors (per cent)					
Site	Tumor type	Group no.:	1	2	3	4	5	6
Prostate gland	Adenoma						1 (2.8)	
Multiple	Leukemia		11 (18.6)	8 (12.7)	6 (10.7)	20 (25.6)	3 (8.3)	10 (24.4)
Total number mice with tumors (per cent)			33 (55.9)	35 (55.5)	25 (44.6)	51 (65.4)	25 (69.4)	27 (65.9)

Table 3. Non-neoplastic Lesions In SENCAR Mice

Site and lesion type		Number of mice with lesions					
	Group no.:	1	2	3	4	5	6
Skin:	focal hyperplasia	1					
	chronic inflammation and hyperplasia					1	
	chronic inflammation, ulcer					1	
	chronic inflammation, fibrosis					1	
	fibrous polyp					1	
Thymus:	hyperplasia	1					
Trachea:	necrosis				1		
Heart:	chronic inflammation				1		
Ear duct gland:	ulcer, granulation tissue		1				
Lung:	chronic inflammation		1				
	pneumonia			1			
	chronic inflammation, fibrosis						1
Lymph node:	hyperplasia	1			1	1	
	abscess					1	
Spleen:	hyperplasia		1				
Liver:	focal necrosis			1			

Table 3. Non-neoplastic Lesions In SENCAR Mice (continued)

Site and lesion type		Number of mice with lesions					
	Group no.:	1	2	3	4	5	6
Kidney:	chronic inflammation		2	2	3	3	1
	scar				1		
	dilated tubules				1		
	chronic inflammation with cystic dilatation of tubules					1	
	focal lymphocytic infiltrate						2
Fore-stomach:	hyperplasia of mucosa		1				
	hyperkeratosis				1		
	keratosis						1
Glandular stomach:	chronic inflammation	1					
	glandular hyperplasia	1				2	2
Duodenum:	focal hyperplasia of mucosa				2		
	glandular hyperplasia and atypia		1				
Anus:	hyperplastic polyp					1	
Mammary gland:	hyperplasia				4		2
	dilated acini						1
	dilated duct with squamous metaplasia		1				

Table 3. Non-neoplastic Lesions In SENCAR Mice (continued)

Site and lesion type		Number of mice with lesions					
	Group no.:	1	2	3	4	5	6
Uterus:	endometrial polyp	2		1	2		
	cystic hyperplasia				2		1
	fibrous polyp						1
	chronic inflammation with squamous metaplasia				1		
	squamous metaplasia of endometrium						1
Ovary:	hemmorrhagic cyst	2	4	3	15		8
Prostate gland:	acute inflammation, necrosis					1	
	chronic inflammation, squamous metaplasia and dilatation of ducts					1	
	multiple fibrous-lined cysts					1	
Seminal vesicle:	cyst					1	
	cystic dilatation					2	
Testes:	atrophy and focal calcification					1	
Total number of lesions		9	12	8	35	20	21

Table 4. Pathology of Clinically Normal Sections

(Groups 4 to 6)

Tissue	Number of Sections			Number of Positive Diagnoses		
Group:	4	5	6	4	5	6
Lung	3	2	1	0	0	0
Liver	8	15	23	0	1[a]	4[d]
Kidney	1	20	20	0	0	1[d],2[e]
Stomach	0	20	12	-	1[b]	0
Skin	4	7	5	0	1[c]	0
Spleen	2	0	0	0	-	-

[a] Hyperplastic nodule.

[b] Papilloma.

[c] Chronic inflammation and hyperplasia.

[d] Leukemia.

[e] Focal lymphocytic infiltrate.

Table 5. Summary Of Frequently Occuring Tumors, In Per Cent

Group Number	1	2	3	4	5	6
Sex	♀	♀	♀	♀	♂	♀
Total tumor bearing animals (per cent)	55.9	55.5	44.6	65.4	69.4	65.9
Lung, papillary tumors	32.2	28.6	16.1	25.6	33.3	39.0
Mammary tumors	5.1	14.3	14.3	24.4	0	22.0
Liver hyperplastic nodules	1.7	1.8	0	0	33.3	2.4
Leukemia	18.6	12.7	10.7	25.6	8.3	24.4
Forestomach papillomas	8.5	6.3	5.4	6.4	16.7	14.6
Number of mice necropsied	59	63	56	78	36	41
Duration of observation (days)	540	600	605	800	815	875

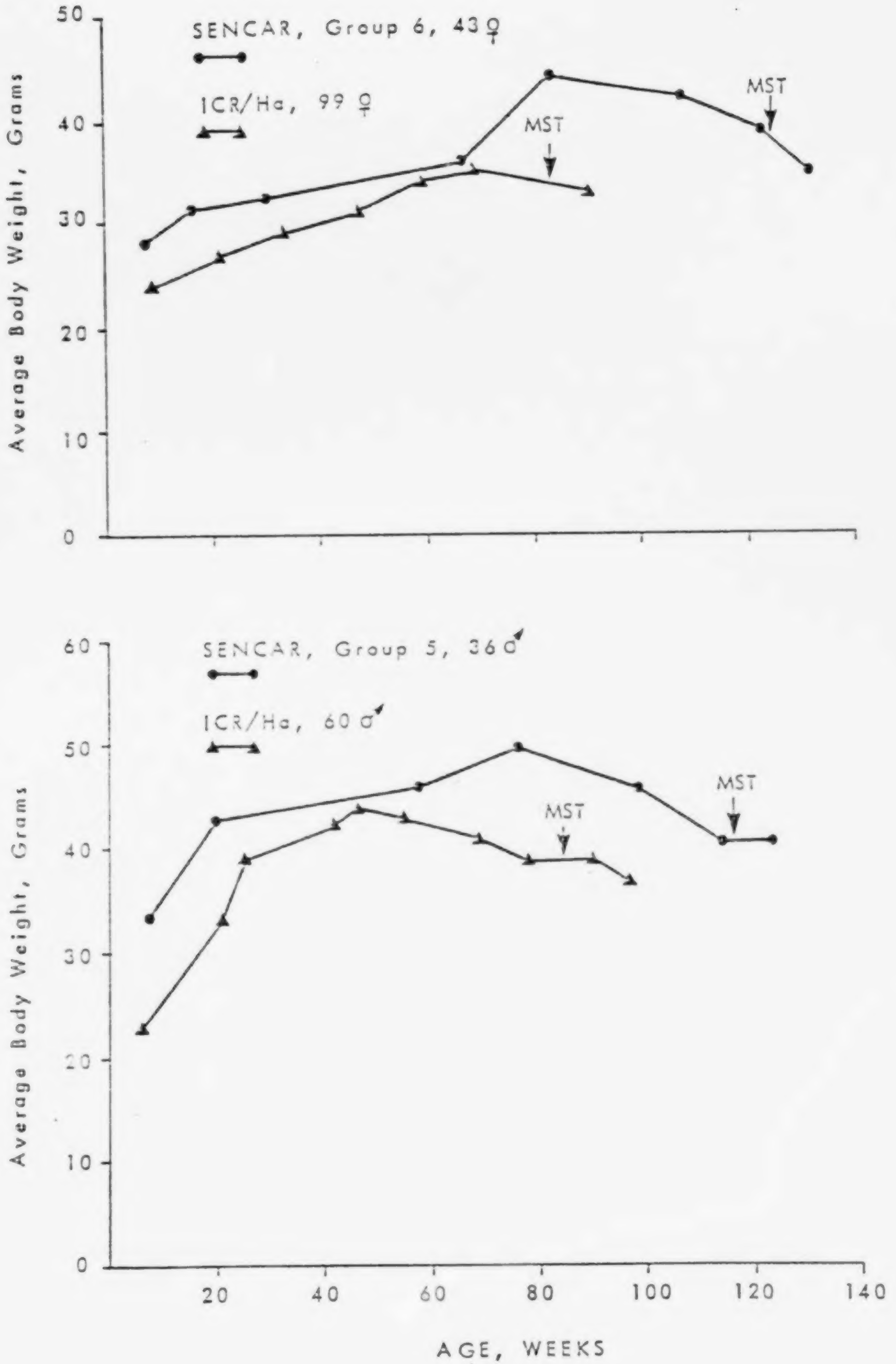

FIGURE 3. Comparison of body weights and median survival times of SENCAR and ICR/HA mice.

PHOTOCARCINOGENESIS AND PERSISTENT HYPERPLASIA IN UV-IRRADIATED SENCAR MOUSE SKIN

by

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Key Words: Ultraviolet radiation, skin carcinogenesis, wound healing, cancer susceptibility, DNA photoproducts, Sencar mice.

ABSTRACT

Susceptibility to photocarcinogenesis has been examined in several mouse strains and stocks including Sencar, CD-1, Balb/c, C3H, C57 Bl, and NZB. Sencar mice are hypersusceptible to tumorigenesis caused by single high dose exposures to ultraviolet (UV) radiation but not by chronic low dose exposures. Sencar mice also exhibit an exaggerated and persistent epidermal hyperplasia in response to UV-induced tissue damage. The persistent hyperplasia is apparently due to a sustained proliferation of the epithelial basal cells, rather than delayed cell differentiation. Sencar mice did not exhibit persistent hyperplasia following other forms of tissue damage (surgical or thermal). In related studies, the levels of thymine dimers induced in Sencar epidermis by UV radiation were comparable to those observed in Balb/c epidermis. In addition, no differences were found in the tissue distribution or persistence of thymine dimers in Sencar and Balb/c skin.

The development of Sencar mice, which are highly susceptible to skin carcinogenesis, has provided a useful model for investigating factors that modulate hypersusceptibility as an approach to the study of mechanisms of skin neoplasia (1-3). Sencar mice are selectively bred to be hypersusceptible to chemically induced two-stage skin carcinogenesis, and this unusual sensitivity has been shown to be an inherent characteristic of the skin of these animals rather than an altered systemic response of the host (4).

We have been investigating certain aspects of photocarcinogenesis regarding susceptibility to tumor induction in mouse skin. A number of experiments have compared strain susceptibility to photocarcinogenesis using single and chronic exposure protocols and these are summarized in Table 1. These experiments have shown that a single treatment of UV radiation (5.8-11.5 x 10^4 J/m^2) from FS 40 sunlamps, without subsequent chemical or physical promotion, results in the dose-dependent induction of epithelial tumors (papillomas and squamous cell carcinomas) in Sencar mice (5). No tumors of any kind are produced in comparably treated CD-1 or Balb/c mice. This finding demonstrates that the susceptibility determinants selected in Sencar mice are not specific for chemical carcinogenesis, but can also potentiate photocarcinogenesis. In contrast, chronic exposure to UV radiation (1.5-2.9 x 10^4 J/m^2 three times per week) is not unusually carcinogenic in Sencar mice compared to Balb/c (Table 1). Thus the exhibition of hypersusceptibility depends on how the UV radiation is administered, suggesting that other factors (e.g. tissue damage) may be involved. Three pigmented mouse strains (C3H, C57BL/6, and NZB/N) are somewhat more resistant than the two albino strains to the carcinogenic effects of chronic UV radiation exposure, based on latency period for tumor appearance. It is noteworthy that the tumors induced in Sencar mice by either exposure protocol (single or chronic) are primarily of epidermal origin and, in this respect, similar to most human skin tumors.

Much of our effort has focused on investigating the biological basis for the hypersusceptibility of Sencar mice to single treatment photocarcinogenesis. One approach we have used to address this question is to examine UV-induced DNA damage in mouse skin to determine whether damage and repair varies between mouse strains. Since several lines of evidence suggest that cyclobutane pyrimidine dimers produced in DNA by UV radiation may be the initial lesion involved in photomutagenesis and photocarcinogenesis, we have produced monoclonal antibodies specific for thymine dimers for use in immunological assays (8,9). Using these assays, we examined the distribution and repair of thymine dimers in the skin of three different strains of mice (Sencar, Balb/c, C3H) exposed to UV-radiation. In preliminary studies, an indirect immunofluorescent assay (10) was used to examine the cellular and tissue distributions of thymine dimers in frozen skin sections; and no major differences were observed among the three mouse strains (unpublished observation). This suggests that the optical properties of the skin that govern penetration and absorption of UV radiation are similar in these mouse strains, and probably do not contribute to variations in susceptibility to photocarcinogenesis among the strains.

A radioimmunoassay was used to quantitate thymine dimer induction and persistence in DNA extracted from mouse skin at various times after UV irradiation (11). Initial yields of thymine dimers in DNA extracted from ear and dorsal epithelium were similar in the two mouse strains tested, Sencar and Balb/c (Table 2). In addition thymine dimer yields measured 24 hours after UV irradiation were similar to initial yields in the epidermis of the two strains. This persistence of thymine dimers in mouse skin is consistent with other studies indicating that murine skin cells are deficient in excision repair of these photoproducts (12,13). A lower initial yield of dimers was detected in Balb/c dermis than Sencar dermis. However, this did not correlate with the preponderance of dermal tumors (fibrosarcomas) arising in Balb/c mice compared to Sencar mice, in which the tumors are mainly of epithelial origin. Thus no clear correlation can be drawn between the distribution, induction, or persistance of thymine dimers in the UV-exposed tissues of these mouse strains and their varying susceptibilities to photocarcinogenesis. This data suggests that Sencar mice do not suffer from abnormalities that alter the levels of DNA photoproducts induced in their epidermis, or the removal of those thymine dimers. Consistent with this conclusion is the recent report (14) that host cell reactivation of UV-irradiated herpes simplex virus by primary epidermal cells from Sencar, Balb/c, and CD-1 mice is similar. These data argue against a defective DNA repair mechanism for the observed hypersusceptibility of Sencar mice to photocarcinogenesis.

It has been postulated (4,15) that untreated Sencar mouse skin contains a population of cells constitutively initiated for tumorigenesis which are resistant to terminal differentiation *in vitro*. In our studies, Sencar mice exhibited an unusual wound healing following single exposures to UV radiation (5 and submitted for publication). These treatments are ulcerogenic and the gross appearance of the healing would was unusual in that it was exophytic and very coarse - somewhat reminisicent of hypertrophic scarring in humans. When we examined this abnormality in detail, an exaggerated and persistent epithelial hyperplasia was evident in the exposed skin of Sencar, but not Balb/c, mice. Epithelial cell proliferation and migration were measured *in vivo* by autoradiography to determine the cellular basis for the persistence of hyperplasia. Twelve weeks after irradiation (when tissue regeneration is normally completed), the rate of epidermal basal cell proliferation in Sencar mouse skin was approximately four times the rate in Balb/c mouse skin that had been identically treated (Table 3). On the other hand, epidermal cell transit times (time for labeled cells to traverse the epidermis) were similar in the two strains of mice. These results suggest that the abnormal hyperplasia in Sencar mouse skin is due to a sustained increase in basal cell proliferation rather than to inhibition of cell differentiation.

In order to determine whether the abnormal hyperplastic response of Sencar skin is specific for UV-induced tissue damage, two other types of tissue damage, surgical and thermal, were examined. Neither full thickness scalpel incision, nor a thermal treatment, which has been shown (16) to mimic UV-induced ulceration, were effective in producing persistent hyperplasia in Sencar epidermis. Thus persistent hyperplasia is not a general response of Sencar skin to all forms of tissue damage. In addition, tissue damage caused by surgical incision administered after sub-carcinogenic exposures to UV radiation did not enhance the tumorigenic potential of the UV radiation. These results are summarized in Table 4.

The precise relationship between sustained basal cell proliferation and hypersusceptibility to photocarcinogenesis remains to be determined.

However, three hypotheses are (i) that sustained basal cell proliferation predisposes Sencar mice to tumor susceptibility by causing repeated replication of basal cell DNA; (ii) that sustained proliferation is an independent manifestation of a genetic defect in Sencar mice which also mediates susceptibility to skin carcinogenesis; or (iii) that sustained proliferation is a pre-malignant state in which the cells are partially transformed.

Table 1. Variations in Strain Susceptibility to Photocarcinogensis

	Latency Period	Anatomical Site	Histological Tissue	Reference
Single UVR[a]				
Sencar	6-12 wks	Dorsum (92%)	Epidermis (88%)	(5)
Balb/c	> 50 wks	-	-	Unpublished
CD-1	> 50 wks	-	-	(5)
Chronic UVR[b]				
Sencar	18-20 wks	Ear (81%)	Epidermis (80%)	Unpublished
Balb/c	18-20 wks	Ear (85%)	Dermis (71%)	(6) & Unpub.
C3H	26-30 wks	Dorsum (68%)	Dermis (77%)	(6)
C57BL/6	26-30 wks	Ear (58%)	Dermis (88%)	(6,7)
NZB	26-30 wks	Ear/Dorsum (50%)	Dermis (64%)	(7)

[a] Single UVR = 8.64 x 10^4 J/m^2 single exposure

[b] Chronic UVR = lifetime exposure (2.88 x 10^4 J/m^2 each) three times per week.

Table 2. Thymine Dimer Levels in UV-irradiated Mouse Skin

Tissue	$(T[]T/T) \times 1000$[a]	
	t < 1 hr[b]	t = 24 hrs[b]
Dorsal epidermis		
Balb/c	0.49 ± 0.05[c]	0.40 ± 0.21
Sencar	0.52 ± 0.07	0.54 ± 0.05
Dorsal dermis		
Balb/c	0.25 ± 0.09	0.23 ± 0.02
Sencar	0.44 ± 0.12	0.38 ± 0.01
Ear (epidermis + dermis)		
Balb/c	0.31 ± 0.22	0.35 ± 0.20
Sencar	0.35 ± 0.13	0.34 ± 0.02

[a] Proportion of thymidines (T) that have formed dimers (T[]T).

[b] t = time after irradiation (1.4×10^3 J/m^2) at which tissue samples were taken.

[c] Mean of two samples ± range.

Table 3. Cell Proliferation in Sencar and Balb/c Epidermis 12 Weeks After Exposure to UV radiation

UVR	Strain	Epidermal thickness 12 weeks after irradiation (range)	Percent basal cells labeled[a]	Transit time (days)
-	Balb/c	1-3 cells	2.9 ± 0.2[b]	8
+	Balb/c	2-5	4.6 ± 0.6[c]	6-8
-	Sencar	1-3	2.6 ± 0.5[b]	8
+	Sencar	5-20	17.5 ± 7.9[c]	6-8

[a] At 4 hrs after injection of [^{3}H]thymidine.

[b] Mean ± S.D. (n = 3); difference between strains not significant.

[c] Mean ± S.D. (n = 3); difference between strains significant $p < 0.025$).

Sencar or Balb/c mice were UV-irradiated (8.5×10^4 J/cm^2) and injected with [^{3}H]thymidine (50 μCi/mouse, intraperitoneally) 12 weeks after irradiation. Dorsal skin biopsies were taken 4 h and 2,4,6, and 8 days later, and processed for autoradiography. Percentages of heavily labeled (>20 grains per nucleus) cells were determined by counting interfollicular cells adjacent to the basement membrane. The approximate time required for labeled cells to traverse the epidermis and reach the keratin layer (cell transit time) was estimated from the position of heavily labeled cells in the epidermis at two-day intervals after injection of [^{3}H]thymidine.

Table 4. Summary of Sencar Skin Responses to Non-Chemical Procedures

	Ulceration	Persistent hyperplasia	Latency period for tumor appearance (wks)
UVR (Single exposure)[a]	+	Severe	6-12[e]
UVR (Multiple exposure)[b]	-	-	> 50
UVR (Chronic exposure)[c]	-	Mild	18-20[f]
Surgical incision[d]	+	-	> 50
Thermal treatment	+	-	> 50
UVR (Multiple) + Incision	+	-	> 50

[a] One exposure (8.64×10^4 J/m^2).

[b] Six exposures (1.44×10^4 J/m^2 each) on alternate days.

[c] Lifetime exposures (2.88×10^4 J/m^2 each) on alternate days.

[d] Unsutured.

[e] Compared to > 50 wks for Balb/c or CD-1.

[f] Compared to 18-20 wks for Balb/c.

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ALLOGENIC TRANSPLANTATION OF NORMAL EPIDERMAL CELLS AND SQUAMOUS CELL CARCINOMAS IN SENCAR MICE

by

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Studies from several laboratories have shown that isolated epidermal cells, either fresh or cultured, may be reimplanted into appropriate host animals. Yuspa et al. (1) developed a silicone chamber that produces a protected area in the panniculus carnosun. Embryonal keratinocyte cultures, transferred into these chambers form an hyperplastic epidermis with normal appendages and hair follicles. A modification of this method was reported by Fusenig et al. (2). They implanted a smaller silicone chamber into a highly vascularized granulation tissue formed by the implantation of a glass disk into the subcutaneous tissue. These authors were able to obtain an organotypic reorganization of normal and malignant mouse epidermal cultures after transplantation to syngeneic or allogeneic recepients (2,3,4).

A similar system was used lately by Mackenzie to protect heterotypic tansplants from skin and oral mucosa (5). The pieces of tissue were placed in a granulation tissue formed with the glass disk technique, and a small polyethlene chamber was used to cover and protect the implant. In this system the skin of the host was closed on top of the chamber, thus completely covering the implant. In this way the graft is protected from infection and dissecation which is one of the problems of the previously described systems.

Tumor transplantation is an important tool in tumor biology. It permits the study of a tumor beyond the life-span of the host and to expand the tumoral mass for different analysis. However, transplantation of mouse skin tumors does not seem to be easily accomplished. Early reports have shown transplantability of a few coal tar (6) benzo(a)pyrene (7) or 3-methylcholanthrene induced tumors (8) into syngeneic mouse. Conversely, 7,12-dimethylbenz(a)anthracene induced skin carcinomas were not transplantable (9). In addition, there are no reports of successfully transplanting skin tumors produced by two-stage carcinogenesis into out-bred mouse stocks such as Sencar. In this report we present a modification of the above described techniques that allow a successful transplantation of fresh and cultured epithelial cells and freshly isolated cells from squamous cell carcinoma in Sencar mice.

Materials and Methods

Preparation of tumor single cell suspensions:

Papillomas and carcinomas were induced on the dorsal skin of SENCAR mice (Harlan, Sprague-Dawley) by using a two-stage chemical carcinogenesis protocol with 10 nmoles of DMBA as the initiator followed twice weekly by a promoter treatment with 2 μg of 12-O-tetradecanoylphorbol-13-acetate (TPA) in 200 μl of acetone, twice weekly. After 10-20 weeks the first papillomas arise and at 20-60 weeks carcinomas develop in 45% of the mice (10). The tumors selected for transplantation were dissected and enzymatically disgregated with a technique developed in our laboratory for obtaining chromosome preparations (11) and short term cultures from skin carcinoma (unpublished results). The tissues were immediately washed in DME medium with Hepes buffer, and the necrotic tissue fragments separated. The sample was later scraped with a scapel thus obtaining a dissociated preparation. The mechanically dispersed tissue was treated with a dispersing solution containing 0.25% trypsin (Sigma Co.) 0.25 type II collagenase (Sigma Co.), 0.1% Hyaluronidase (Sigma Co.) 0.5% bovine albumin in a modified Hanks' solution with Hepes buffer (pH 7.4) prewarmed at 37°C. The tissue is stirred slowly at the same temperature with a magnetic bar. The first 10 min. wash, enriched in squamous, necrotic and inflammatory cells is discarded. Two more washes of 20-25 minutes are processed separately immediately after being obtained. The collected supernatant is filtered through a steel screen mesh. The enzymatic treatment is stopped by cold DME medium with 10% fetal calf serum. The resulting material is spun down at 1000 RPM and the pellets are resuspended in fresh medium.

Preparation of newborn single cell suspension

Single cell suspensions of new born epidermis were obtained by the method described by Yuspa (1). Briefly, the skins were floated in 0.25% trypsin overnight at 4°C. Dermis was separated from the epidermis using sterile forceps and tweezers. The epidermis was washed in cold tissue culture medium with 10% fetal calf serum, minced with scissors and stirred in the medium for 30 minutes. The cell suspension was filtered through a nylon mesh, spun down and resuspended in fresh medium without serum.

Chamber preparation

Silicone chambers were manufactured in our laboratory as described in Figure 1. Medical grade silicone 3/8" x 1/2" tube (Dow Corning) was cut in pieces of 1 cm and one end closed with circular silicone pieces obtained by cutting medical grade silicone sheeting 8" x 6" x 0.4 mm (Dow Corning). Both silicone pieces were glued together with medical adhesive silicone type A (Dow Corning).

Cells and tissue implantation

Preparation of a suitable graft bedding was obtained by the subcutaneous implatation of a glass disk as described by Fusenig et al. (2,3). Between 2 to 3 weeks after the implantation, the disk was removed from the animals by an incision made over the glass disk. A pocket of granulation tissue which surrounded the glass was formed. The chamber was placed inside this pocket and the skin sutured with clamps over the chamber. After 24-48 hours cells suspension were injected through the skin and into the chamber usng a syringe and a 25G needle. Approximately 1-2 x 10^6 cells resuspended in 0.4 ml medium without serum were injected in each chamber.

Explants of tissues were grafted in the chambers directly on the granulation tissue immediately after removal of the glass disk (5). The explants covered by the chambers and the skin closed over the chambers as described previously.

After 2-6 weeks the animals were sacrificed, the chambers dissected out from the tissue bed and the tissues fixed in buffered neutral formalin, embedded in paraffin and stained with hematoxylin and eosin.

Results and Discussion

The method described here to implant normal and tumor cells into SENCAR mice is essentially similar to the technique described by Mackenzie (5). However, we have developed a chamber which can be easily prepared in the laboratory and seems more suitable for cell growth. The original polyethylene chambers described by Mackenzie, were excellent to support the growth of full tissue explants, but in our hands were not adequate for the growth and tissue reorganization of single cell suspensions.

Explants of skin and vagina were grown in the chambers for a period of 4 weeks. Even when some of the explants exhibited signs of immunological rejection, most of the explants looked healthy after three weeks (Fig. 2 and 3). Most of the explants showed a large outgrowth of the epithelial cells covering the granulation tissue.

Isolated epithelial cells from newborn mice injected into the chambers formed an almost normal epithelium within two weeks (Fig. 4). Rudimentary hair follicle and glands were observed in all the cases. The presence of skin adnexa was previously described by Yuspa (1) and has been ascribed to the inductive properties of dermal fibroblasts (4). In our case, the presence of skin adnexa suggests fibroblast contamination of our epidermal preparations. The possibility that the new formed skin can be produced by an outgrowth of the host skin can be ruled out because, a) reparative epidermal growth is not usually accompanied by adnexa, b) there is no continuity between the skin and the newly formed epithelium, c) chambers which have not been inoculated with epithelial cells do not produce epithelium. We also ruled out the possibility of the epithelium coming from remanents of hair follicles because, a) as previously stated empty chambers do not produce epithelium, and b) we have never observed histologically hair follicles trapped in the granulation tissue.

Cells from skin squamous cell carcinomas were isolated by a method developed in our laboratory and used successfully to obtain tumor karyotypes (11). It has also been used to obtain viable cells for short term culture (Conti et al., unpublished results). The injection of this cell suspension produced the growth of neoplastic tissues within two weeks (Fig.5). The tumors were well differentiated squamous cell carcinomas resembling the original neoplasia. When tumors are allowed to grow over the fifth week they start showing signs of rejection.

The possibility of implanting epithelial cells in allogenic host was previously explored by Fusenig and co-workers (2,3). They found that pure epithelial cells are not rejected when implanted in chambers up to at least seven weeks. In a similar way, we have been able to grow normal and tumor cells from the outbred SENCAR stock for a minimum of 5 weeks. Some of our preliminary experiments indicate that beyond the 5th week rejection of the graft, especially tumor cells, may take place. However, since one of the great advantages of the chambers assay is the fast cell growth, a five week period seems to be adequate for most tumor transplantation studies. The chamber system also appears very promising as a fast assay for malignant transformation of cell cultures. Preliminary studies from our laboratory have shown that keratinocyte transformed *in vitro* form an epithelial structure with areas showing invasion within two weeks.

Summary

A method used in our laboratory for the transplantation of single cell suspension of epidermis and skin carcinomas is described. Silicone chambers were placed into a subcutaneous granulation tissue formed by the implantation of a glass disk. The skin was closed on top of the chamber using Michael autoclips. Cells were injected 24-48 hrs later through the skin using a syringe and needle. The neoformation of epithelia with adnexa was observed when newborn epidermal cells were injected. When a fresh cell suspension of squamous cell carcinoma was used a typical differentiated carcinoma was formed within 2-4 weeks. However, after 4 weeks some of the grafts showed mild signs of rejection. The technique described here is a useful system to transplant squamous cell carcinomas and can also be used as a rapid assay for malignancy.

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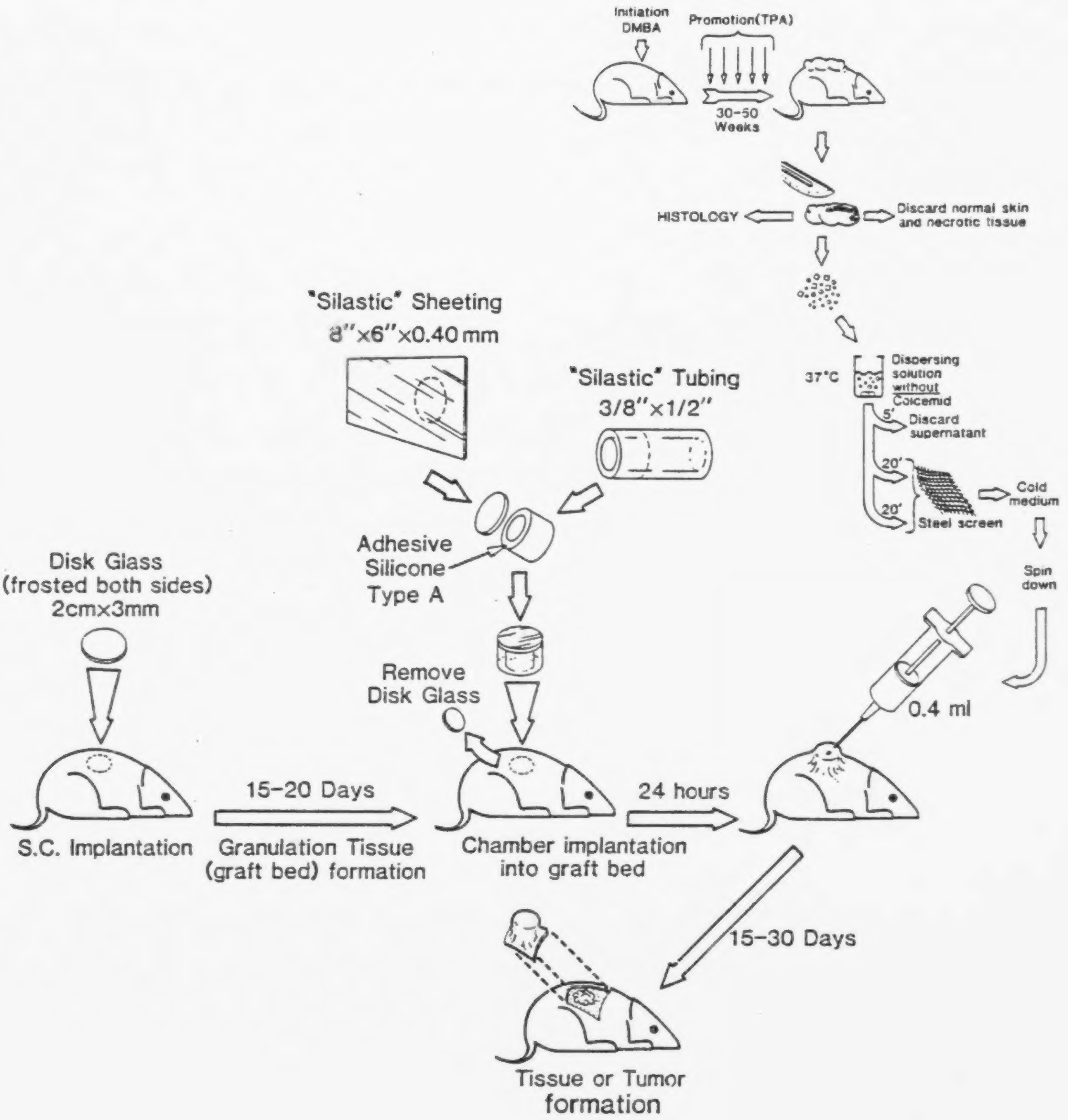

Figure 1: Schematic representation of the technique used for the transplantation of skin tumors in SENCAR mice.

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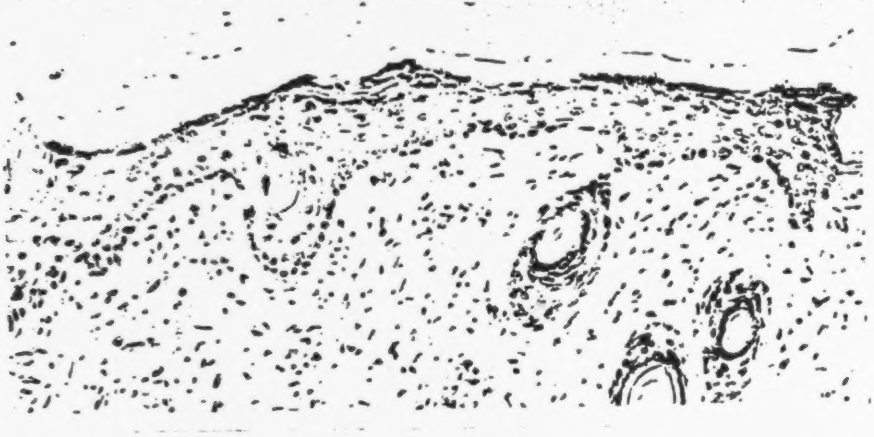

Figure 2: 3 week old transplant of SENCAR mouse skin implanted in 6 week old SENCAR mouse. Hematoxylin and eosin 70 X.

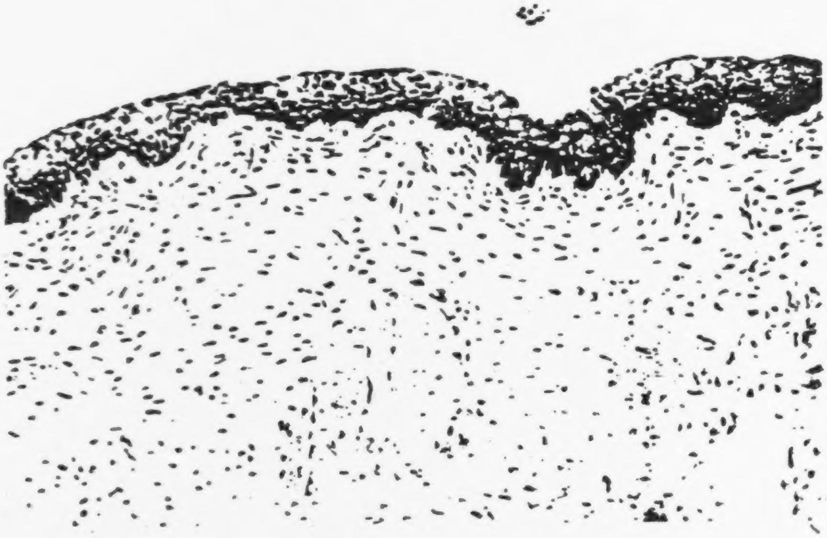

Figure 3: 3 week old transplant of SENCAR mouse vagina in a chamber. Hematocylin and eosin 70 X.

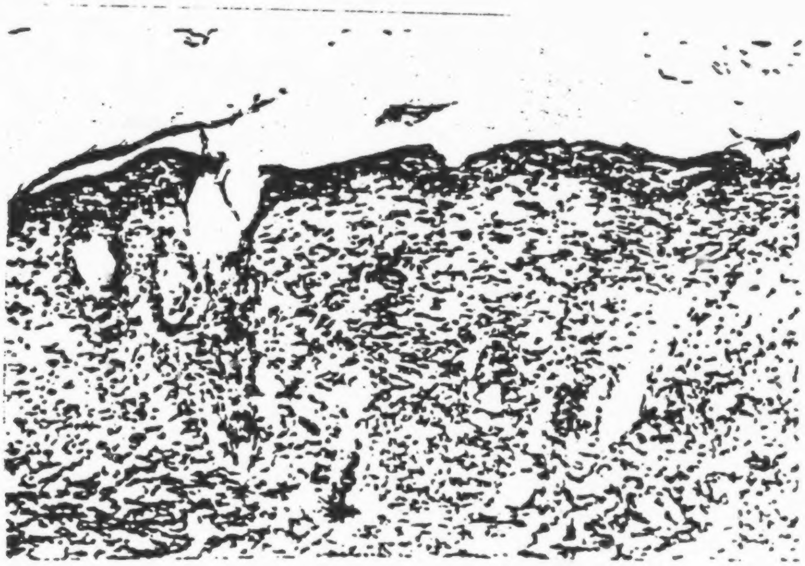

Figure 4: Reconstituted epithelium in chambers injected with 10^6 cells from SENCAR new born epidermis. Hematoxylin and eosin 70 X.

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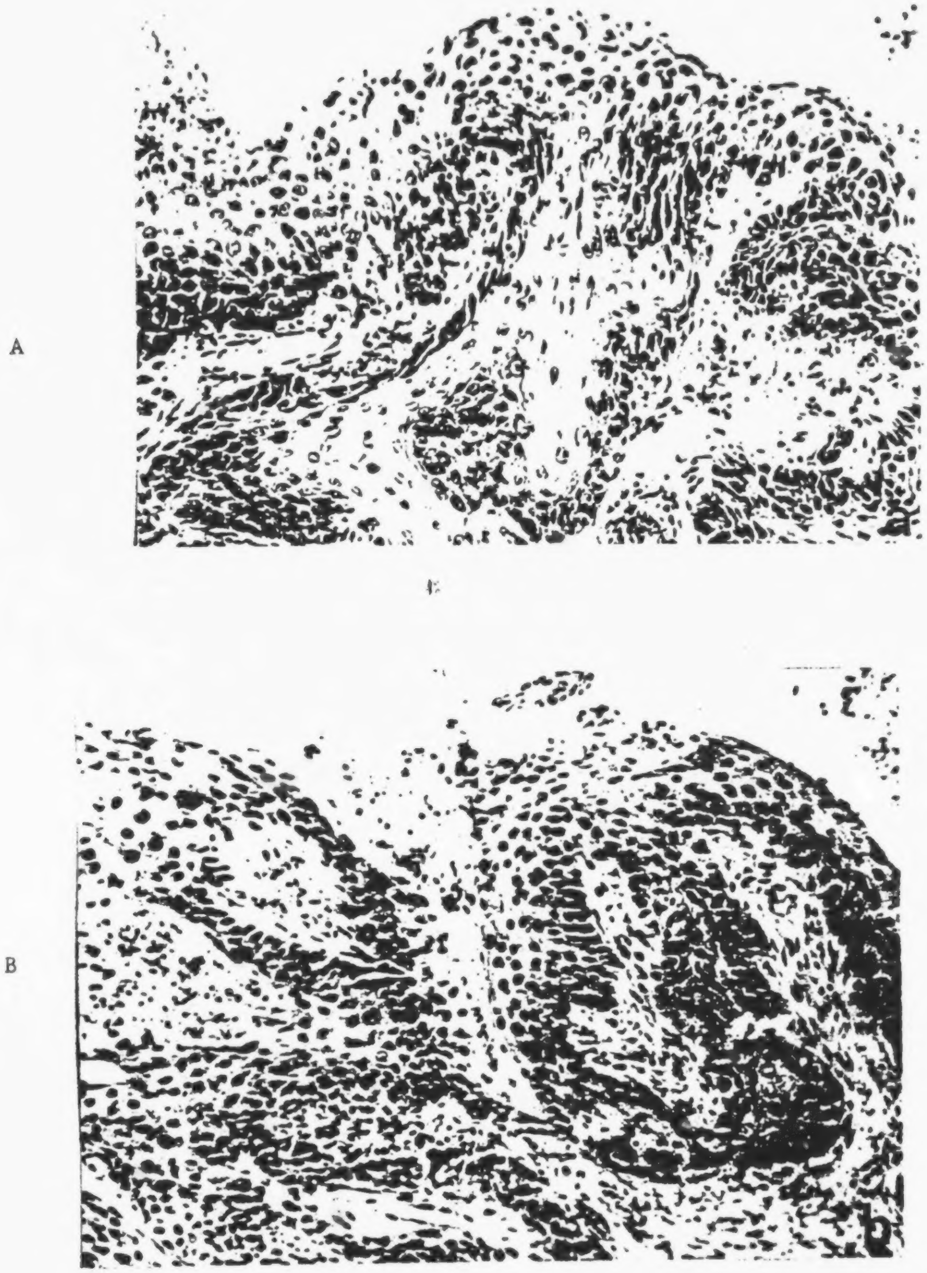

Figure 5: a) Squamous cell carcinoma induced in SENCAR mice by a two-stage carcinogenesis protocol. Hematoxylin and eosin 100 X.

b) The same carcinoma after being dissociated and grown in the chamber for two weeks. Hematoxylin and eosin 100 X.

Dr. Richard J. Bull (No. 14). There are, and I think maybe some of the questions that we might discuss this afternoon might be discussed from anyone of these particular perspectives. I think it may be important we run through, at least recognize, that there are some people in the audience that have concerns, regulatory agency, they are looking at the Sencar as a screening system. The questions they are asking are going to be somewhat different than the questions that the people from the National Cancer Institute or some of the people from academia might be asking and I think that they make it an even more interesting discussion if we recognize that. So, I think I won't say much more, I will just open it up to questions from the audience or any points that the speakers feel that need to be raised early.

Dr. James Strickland (no. 27). I think there is one thing that is extremely important that be made very clear. That is, the Sencar mouse was derived to be sensitive to skin carcinogenesis by a certain protocol, and I think our subsequent work has indicated that the mouse is sensitive to skin carcinogenesis by a variety of other agents different from the ones used to derive the sensitivity, but still the biology of the Sencar mouse is defined for sensitivity to skin carcinogenesis. People who are interested in using the mouse as a test system should recognize that, as far as I can tell from what I know in the literature, and what has been presented at the meeting, there is no good biology for increased sensitivity of the Sencar mouse in any other organ than the skin.

Dr. Judy Stober (No. 40). There is a general comment that I want to make. Considering the issues I addressed in my talk and the issues raised by other speakers in their presentations, it has become more evident that in order to objectively evaluate this assay, it is absolutely essential that the person doing the research define the criteria by which they are judging the response in the Sencar animal. We need to know exactly how they are determining that a lesion is a papilloma or a carcinoma. Is it being scored objectively by the gross lesion or is it being done microscopically? In order to evaluate results and compare results across time or between researchers it is essential to know this. Also it is very important to know how the descriptive statistics reported are calculated. If we don't know how it was calculated, we don't know how to interpret it or how to compare it to other results. I just wanted to emphasize these issues because I think that they became more evident after listening to the presentations the last two days.

Dr. Richard J. Bull (No. 62). One of the things that I wanted to make a particular point of is in the context of using the Sencar mouse as a screening system and I think that the point Dr. Strickland made is well taken. There is, obviously, some problem in using it across the board as a screening technique. Its use is obviously, from our results, restricted to certain classes of compounds that impact certain target organs. Given that restriction one has to think of it, in this particular assay system, as a screening system in the context of other systems. I think that is one way to get around it. But I would like to move that question along to a point that Steve Nesnow brought up in his presentation around the use of Sencar results. The agency made, in terms of trying to deal with the diesel exhaust situation, is that is a step in saying well not only is the system useful in a qualitative screening situation, given certain restrictions perhaps on the class of chemical carcinogen we are dealing with, perhaps it would have some utility in making some estimates of risk that are as valid, or nearly as valid, as are employed with long time carcinogenesis, two year feeding studies, basically. I don't know if you care to comment more on that Steve, or not.

Dr. Stephen Nesnow (No. 85). What we were faced with about three or four years ago was to come up with a way of evaluating the potential hazards. In particular, automobile exhaust, particularly the diesel exhaust, in relationship to what we were currently exposed to as a population in terms of gasoline exhaust. That is, there was a tremendous push to dieselize the automobile fleet, and the production of diesel exhaust which contains tremendous amounts of particulate material, more than does gasoline exhaust - how would that affect human health? And we knew from previous studies and also from studies that were done here in Cincinnati on inhalation, that these complex mixtures, particularly automobile exhaust, were weak carcinogens and would not induce respiratory tumors in a variety of species. So we had to come up with some way of evaluating their comparative activity, and we chose the mouse skin model because, obviously historically, mouse skin has been a very good and sensitive system mainly for polycyclics but for some other classes as I pointed out. But particularly for complex mixtures it seems to be a very unique way of obtaining good dose response data. We picked Sencar, obviously, because we were looking for increased sensitivity for a potentially weak carcinogen and that is exactly what we found. Of course, we found a variety of diesel exhaust to have different activities and found some to inactive to some moderately active and we found that if we used enough animals and enough doses that we could get good dose response data, good quantitative data, and it was reproducable and we could evaluate these mixtures on the incidence of tumors that they induced on this animal. I think we could have done the same thing with other strains of animals, but we picked Sencar because we were looking for a wider response. We have been able to take this data and, as I mentioned in my talk, to compare it to data obtained in other short-term tests systems and other in vivo systems, particuarly as I mentioned the human epidemiology and we have taken these same mixtures and looked at their comparative activity, say in the Ames test, or in mouse lymphoma gene mutation assay, cell transformation assays and so forth. One system that seemed to parallel in terms of potency the human experience, for human carcinogens, human complex mixture carcinogens, was the mouse skin model. Now why that is I don't know. But there was a direct relationship as I mentioned between mouse skin tumor initiating activity and complete carcinogenic activity of these mixtures which included coke oven emissions, roofing tar emissions and cigarette smoke. There was like a 1,000-fold variation in response in humans and about an 800-fold variation response in mouse skin. This kind of data, we are presently trying to utilize in a perspective way by taking mixtures which are of more current interest, say for example, wood smoke emissions which is of great interest to EPA, and to look at their activity on mouse skin and to compare it with these reference carcinogens from which we have both mouse skin data and human epidemiology.

Dr. Jerry Ward (No. 140). Two questions concerning the testing of chemicals on mouse skin, especially Sencar. Does anyone here have any results that show that the Sencar mouse developed tumors and other strains did not from one type of either initiator or promoter. We had a study with a phthallate in which we did CD-1 mice first, in a continuous promotion two-stage system and then a three-stage system with two stages of promotion TPA followed by the phthallate and it was positive in the Sencar and negative in the CD-1. We went up to 40 weeks in the CD-1. You can always say if you waited longer you might get some more tumors, but does anyone have results that show consistently with one chemical or more that it is positive in the Sencar and not in other strains. And then questions number two, is if that is true for some chemical, what does it mean? It was picked up in a more sensitive strain and that strain may be abnormally sensitive and not relevant to the rest of the mouse world much less the human population.

Dr. Stepehen Nesnow (Mo. 156). In my talk I did present several examples of that, but they were extreme examples in which we had the same chemicals looked at as mouse skin tumor initiators in C57 black and Sencar. Since that work came out and Tom and his group have been able to investigate these findings in which they were inactive on C57 black and active in Sencar. It seems to be a deficiency in the blacks in responding to TPA as a promoter. As when they use benzoperoxide they did get activity and as a complete carcinogen C57 black at least with benzopyrene and I think with MNNG, the C57 black and the Sencar are somewhat equivalent in activity when you do it as a complete carcinogen. So, I think the enhanced sensitivity, at least in our hands and the literature that I read, seems to be in the area of certain polycyclics, not all polycyclics, but certain polycyclics, definitely DMBA and somewhat with the benzo-a-pyrene. I don't really know what about 3-MC in the comparison. But other chemicals, like MNNG, it is not more sensitive.

Dr. Richard J. Bull (No. 178). To continue this subject for just another second, I am a little uncomfortable, I think the strength of the work that you did with diesel exhaust is the fact that you had the epidemiology data to fall back on and say you had a pretty good relationship for these three other conditions and this this is a similar condition. I am not sure how broad you can apply that to complex mixtures. In view of the fact that there seems to be a fairly high false negative rate for compounds that would ordinarily impact other target organs and so on and so forth. For example, based on our results with the aromatic amines, I would be very hesitant to use the Sencar mouse to judge the hazards from a dye effluent, if you will. I think it has got definite limitations in the complex mixture. I think you have to know your sample.

Dr. Stephen Nesnow (No. 191). You have to know at least what the major components of your mixture are at least in terms of class analysis, whether they are polycyclics or whether it is basic or acidic or so forth. We have done several aromatic amines on mouse skin. We have done beta-naphthylamine and we have done 4-aminobiphenyl and they are totally inactive. At least when you apply it dermally and promote it dermally. You have to be careful. You have to know what class of chemical you are dealing with before you select any system whether it is mouse skin or Ames or lymphoma or whatever.

Dr. Richard J. Bull (No. 199). If I could get off that subject directly. Unless somebody has something elese they want to jump in and bring up in this particular area. I am kind of dwelling on the regulatory thing, and hope we can move more toward the basic stuff later. There is one area that I think that is kind of central to the use of the Sencar mouse as a short-term screening situation and that is the circumstance of regression and progression. Particularly the progression to the malignant stage and I was very happy to see Dr. Hennings presentation and see that someone was trying to address that question in a basic science sort of way. May I put Dr. Hennings on the spot. We didn't talk about this before hand, but it seems to e that the work that you were doing would suggest that there are two hits involved in complete carcinogenesis - at least, perhaps more. Do you see from your seat in the National Cancer Institute how that might influence the regulatory postures of regulatory agencies with respect to risk assessment models and that sort of thing.

Dr. Henry Hennings (No. 220). Well, I think one thing that I want to emphasize about our work is that TPA does not seem to be involved in the conversion process from papillomas to carcinomas. That may not be typical of tumor promotors, so as I said in my talk, I think the role of TPA is to give us papillomas which then have a certain chance of progressing to carcinomas. Maybe with each cell division you have a chance of that mutation happening spontaneously because it does seem to happen spontaneously as well as when we treat with genotoxic agents like urethane or 4NQO. I don't know whether I am answering your question.

Dr. Richard J. Bull (No. 237). I was particularly interested in the slide in which you were dealing with the higher dose of TPA that gave you the higher yield and they seemed to regress and with the lower dose, you seemed to get a plateau of 4 or 5 papillomas per animal yet you seemed to get a better conversion. And I am curious. It seemed like the regression, if you were to extrapolate that, the one weakness you might see in the use of gross papillomas count is that all papillomas do not necessarily, well I think that's a given, not all papillomas progress to carcinomas, but you are saying there are some papillomas that progress preferentially.

Dr. Henry Hennings (No. 249). There is, you know, a big heterogeneity among papillomas which that experiment in particular showed very clearly and I think a lot of the TPA dependent papillomas have nothing to do with carcinomas. It seems to me that, if you were basing some regulatory decisions on papilloma counts, I am not sure that makes sense. I think you would be better to base them on real carcinogenesis, the production of carcinomas..

Dr. Richard J. Bull (No. 258). Well, I don't know if you noticed the one slide in my presentation where I was trying to ratio the papillomas to the carcinomas, but in essence if you look at the shape of that curve, it argues very much the same point that you had. That if you had a relatively low papilloma yield, you have got a higher conversion rate than you did as you got out to 10 or more. In fact, we eliminated anything above 10 on our particular slide. There is definitely a curva linear relationship. In our case we were looking at all papillomas and all the carcinomas as you heard Gary describe, so that ratio of conversion certainly changed as the papilloma yield increased.

Dr. Henry Hennings (No. 267). Of course there is a limit to how many carcinomas an animal can get before it dies. So if you have, say twenty or thirty papillomas, a mouse is not going to get more than four or five carcinomas. At best here if you are dealing with high papillomas incidence, you can't have a terribly high conversion.

Dr. Richard J. Bull (No. 272). That's the reason why we limited ours to an area of five to ten.

Dr. Stephen Nesnow (No. 273). I think there is some data in the literature for DMBA and BP which suggests that papilloma multiplicity, the number of papillomas per animal, and in the tumor initiation protocol, and the carcinoma incidence, the number of animals with carcinoma, and the complete carcinogenesis protocol for those agents seem to parallel in terms of dose response. So that is also something. There are three, I mean you can measure carcinomas as a result of conversion of papillomas in tumor initiation. And that may also somewhat occur in the complete carcinogenesis, but I think there is a parallelism when you are measuring carcinoma formation and complete carcinogenesis and papilloma multiplicity in tumor initiation.

Dr. Richard J. Bull (No. 286). To use as a screening mechanism, to make it economically feasible, you almost have to deal with the papilloma if you are really going to use it as a screening situation and that's really the reason for pressing that issue. I have held the pathologist in check here for a couple of minutes, but I know Gary Knutsen has some very interesting things that he would like to bring up for the pathologist to hash over and interact with the toxicologist on, if you would like to start any discussion along that line it would be appreciated.

Dr. Gary L. Knutsen (No. 296). Just a general question. Maybe a block buster, I don't think so. We saw some lesions today, a lot of lesions. We saw them from the NCI colony and from Sencars at NCI, Sencars from Harlan Sprague. I would like some pathologist to comment that were observing these lesions as we were showing these, and that sort of thing, if they got any feel that there is any spontaneous or even helped pathology, induced pathology, that was occuring. That should give anyone concern if there is a little bit of difference in the responsiveness of the two animals. Who would like to grab that? No one.

Dr. Andres Klein-Szanto (No. 306). The only thing I can say about that is that in recent experiments done with Harlan animals, we have seen a much higher incidence of tumors and a shorter latent period with those animals than with the original Oak Ridge animals which, as matter of fact, are similar to the Frederick colony. So, Harlan, by some mysterious phenomenon is different. That is the only thing I can say - the response is different.

Dr. Henry Hennings (No. 315). Is that papillomas or carcinomas?

Dr. Andres Klein-Szanto (No. 317). Both.

Dr. Richard J. Bull (No. 329). Let me repeat that. The original Oak Ridge stock was more sensitive.

Dr. Andres Klein-Szanto (No. 320). The original Oak Ridge and the actual Frederick is less sensitive than the Harlan.

Dr. Richard J. Bull (No. 323). Are there other differences between those two sources of animals that we ought to be concerned about? Are they drifting apart such that they are not the same beast anymore?

Dr. Andres Klein-Szanto (No. 330). The other thing I have heard, but I cannot swear that this is correct, is that the Harlan data were not very homogenous so once they were more sensitive than the original stock, and in another experiment they were more or less the same and in the third experiment they were less and so on, but that is just what I heard. Just hearsay. I am not sure.

Dr. Richard J. Bull (No. 336). We had some data to that affect. Steve, is yours an NCI stock or Harlan stock? You have been getting from Oak Ridge primarily, right? (Nesnow - Oak Ridge). Bull - I didn't see our variability being that much more than.

Dr. Stephen Nesnow (No. 340). Speaking of rumors, I heard the rumor that there was a weight difference in the two different stocks.

Dr. Andres Klein-Szanto (No. 342). Correct. The Harlan are larger.

Dr. William Baird (No. 344). I think one of the biggest questions with both of these stocks is are people going to continue to select them? Because I think otherwise you will get a drift with time. Boutwell sent us some data right before he left for Japan where he had compared a strain he kept which was continually selected versus I don't know which of the commercial Sencars and there is not a big difference, but there clearly is a difference in sensitivity. I think whoever maintains these is going to have to put into their protocol that maybe once a year or once every two years, they go back through this DMBA TPA selection. Otherwise I think you will keep getting an increase in variability and you will also get a decrease in sensitivity.

Dr. Richard J. Bull (No. 355). That brings up a question I heard Dr. Strickland talk about the other day and that had to do with the fact that maybe we shouldn't be comparing the very unsensitive to the very sensitive. I kind of like that point. One of the things that sticks out in my mind about the Sencar is it is apparently selected in terms of its sensitivity for both initiators and promotors. It seems like if you have at least a bivariate selection there as opposed to a univariete selection there is likely to be more than just one reason why the Sencar does exhibit an increased sensitivity in the initiation promotion experiments at least. Does anybody else have a comment on that? I heard Bill Baird had a comment on that yesterday. I didn't hear any body else pick up on it. Is there some way we ought to look for more subtle differences between strains or is that even a feasible thing to do? Nobody wants to touch that one. Bill.

Dr. William Baird (No. 372). I guess I will touch it just by asking the question how many strains do you want to maintain? These are not strains, as Tom said, these are stocks so in order to maintain a reasonable growth number in birth size, you are talking about at least four groups is the way Boutwell did it. And now if you are keeping four groups which are selected for promotion and four which are selected for initiation, your chances for getting a mix-up in the breeding become much, much higher. Based upon some experience we had at Wisconsin, I think I would rather eliminate that possibility and have one set and deal with that. I think Tom's idea of an inbred strain should solve a lot of these problems. I think that is really the way to go.

Dr. Richard J. Bull (No. 379). Do I hear any more? I keep turning my back on the audience. I would appreciate any questions that are out there, if you have any please come to the microphone. We don't want to monopolize the time up here. Dr. Ward.

Dr. Jerry Ward (No. 383). It seems now that EPA uses more of the Sencar mice than anybody in the world and I mean 11,000 in a couple of years, that's a lot. Could someone say what the plans are for the future or the ongoing studies. Is this a continuing thing?

Dr. Richard J. Bull (No. 387). I am no longer in a position to answer that question. I can talk about why we began to do work with the Sencar. It started out a bit of an accident. We got some results on some drinking water, disinfected with chlorine, which we have never been able to repeat, but it got us sunk into the Sencar to some extent as a screening system. And that's probably the reason why, if you really go back into the reason why we started using it, that's it. Now, it's become, because of our history with it, I am sure that there is going to be some continuation of that. The extent, as I say I am not the person to make that decision any longer, but I suspect there will be some level of Sencar use as a screening situation because we found it fairly useful in our program at least for some direct acting compounds that are generated as by-products of chlorination. Now, Steve could maybe comment on how dedicated the people at Research Triangle Park are to the Sencar system.

Dr. Stephen Nesnow (No. 406). Well, we are dedicated, but sometimes our dedication is overruled by our management. We have a major investment in the Sencar in terms of, as you have already heard, in terms of air pollution and we are definitely continuing looking at using that system for comparative purposes and also for some pure chemicals which would seem to benefit from that kind of analysis. It is no where near the size of the program that Dick and I used to have, which was actually on the order of something like 40,000 animals. It is much, much smaller. But there will be a continued effort. It seems from this symposium there needs to be more work done in types of dosing, pathology, sensitivity. There needs to be more work done. As a standard screening system, it is still very valuable right as it is and I think it will be continued, but I understand there are other governmental agencies that are interested in supporting it, particularly NTP to some extent and I guess I will leave it like that.

Dr. Richard J. Bull (No. 428). Sometimes I think that we in EPA kept it alive. I don't know if that's good or bad because for a long time, I think that the EPA, not to brag or anything, I think the EPA's support is what kept it going at Oak Ridge. That may be an overstatement of the case, I really don't know. We were by far the biggest users and the difficulty I think and maybe, Skip, I don't know if he would care to comment on your plans with the Sencar. You had mentioned a few things about at least evaluating it for further use.

Dr. William Eastin (No. 441). My primary interest in even attending was to see what everybody else was saying about the Sencar mouse because we are interested in adopting a strain for initiation promotion studies in the National Toxicology Program. Some of the things I have heard have made me worry a little bit about what the most appropriate controls are going to have to be. I have talked to some of the pathologists and we really don't have a good data base on untreated Sencars. I have seen some information here that I have never seen before. One of the strains that we are comparing the Sencar mouse with is the B6C3F1 which we use routinely in the program and we have a huge data base and we are a little reluctant to give up that kind of information. I guess a good question to ask the panel is what do you consider the most appropriate controls, in a routine, especially given that there may be differences between the two major suppliers right now. Do you need to have them clipped with no treatment? Naturally you need a vehicle. You began to expand your numbers of groups tremendously, but it seems like it is an obvious question. We need to build a data base and everybody ought to be doing it the same way. Any comments on that?

Dr. R.M. Kovatch (No. 466). I might make a comment to that regard. When the B6 started in your program they had room controls. They have eliminated those over the years. They feel it is not a requirement, and it might be the same with the Sencar model. I think you ought to start with adequate numbers of controls and downstream possibly in a few years you can eliminate those room controls from your experiment.

Dr. Richard J. Bull (No. 477). I was going to comment to some extent. Now this is not strictly an untreated control. One of the things that we very rarely show in the slides, because our interest is primarily on testing the compound as an intiator, we have always run non-promotive controls. Now they have received acetone, as a vehicle control. There's a lot of data there. You have to recognize they have been treated with acetone and they have been shaved. So, that's something we have not really looked at systematically in isolation from the rest of our data because most of our interest is obviously focused on the initiating activity. So that is some place that, talking to Merrel Robinson and Gary Knutsen, if you needed to see that kind of data, I think that kind of data, at least for the acetone-treated animals would give you a feel for what the background is. It may not be exactly what you want. Before you invested a lot of money in generating background information on 10,000 animals, at least it would be a step in that direction.

Dr. Richard W. Niemeier (No. 498). I have been working with complex mixtures for quite a few years. We have used exclusively the C3HHEJ for assessment of complex mixtures. This was really a holdover from when I was at the Univeristy of Cincinnati, with Eula Bingham, since we did a lot of oil samples with C3HHEJ. Recently, I just completed a study with CD-1 and C3H using asphalt fumes and coal tar pitch. We found that asphalt fumes were highly carcinogenic. Now we are going on to do some more work with the asphalt fumes with various fractions and one of the things that we are easing into is the use of the Sencar simply because of their increased sensitivity. We want to compare this to the C3H model we have been using over the years. Those are our future plans.

Dr. Andres Klein-Szanto (No. 519). I would like to return to the previous question regarding controls. I think probably from the proceedings of these meetings today and yesterday, and from what has been said here this morning, everybody will find quite good and complete data on the baseline pathology of these animals and I think that most of those data are in regards to the original stock, Oak Ridge and Frederick, and do not include the Harlan. None of us have presented Harlan data. At least I don't have any on that type, but I think the other original stock of animals is pretty well characterized and we know very well the pathology. We had, as I said this morning, an avalanche of data on that type of lesions.

Dr. Gary L. Knutsen (No. 538). Our mouse was totally Harlan.

Dr. Andres Klein-Szanto (No. 539). Yours was.

Dr. Richard J. Bull. Well, we have both then except our has got acetone treatment for the most part.

Dr. Gary L. Knutsen (No. 540). I am very uncomfortable that we have anything that resembles a baseline on anything at this point.

Dr. Richard J. Bull (No. 543). It would seem like that would make a good comparative base nonetheless. You had a couple, almost 300 animals, of non-treated animals as I recall in your work.

Dr. Andres-Klein Szanto (No. 547). We have 500.

Dr. Richard J. Bull (No. 548). It was a fair number.

Dr. Andres-Klein Szanto (No. 550). Those are untreated Oak Ridge animals. But Jerry has some data on normal, untreated, animals too.

Dr. Jerry Ward (No. 553). We have 12-month-old animals right now, in fact last week. And then we have 2-year-old animals, next month we will be sacrificing them in cooperation with Dr. Paul Strickland, at Frederick, who has done UV carcinogenesis studies, so we will be able to compare those. But I think if one institute is going to use one particular strain, they should characterize that one particular source of the stock. They should characterize in that facility with the same feed and the same environment, caging, hair clipping and all that. They should characterize their controls and then you will be able to compare it to other people.

Dr. Gary L. Knutsen. Dr. Klein-S zanto, you mentioned this morning, and showed very dramatically the amyloid in the liver and spleen and also Dr. Kovatch showed amyloid liver and spleen. I notice that Dr Ward did not mention this. He mentioned it in the thyroid and nasal cavities, and things like that. We have formally been talking about what appeared to be, at least superficially, quite difference in amyloid between these. You are saying the animals you were showing slides of were actually Oak Ridge. Are you currently getting Harlan animals as well?

Dr. Andres Klein-Szanto (No. 583). We did but we are not doing that any longer. We are getting them from Frederick.

Dr. Gary L. Knutsen (Mo. 584). So the animal you were showing was....

Dr. Andres Klein-Szanto (No. 585). Were Oak Ridge. Original Oak Ridge animals which have never seen the soil of Texas.

Dr. Gary L. Knutsen (No. 587). In other words then, the amyloid we are seeing, the amyloid you are seeing, we do have two sources there.

Dr. Andres Klein-Szanto (No. 590). Of the animals I have seen are only Oak Ridge animals. Those were animals which were treated in Oak Ridge, analyzed partly in Texas, but they were all treated and originated in Oak Ridge.

Dr. Gary L. Knutzen (No. 597). My concern was that I saw some differences. They were very subtle between the kidney lesion and of course amyloid hooked into this amniologic phenomenon, and I was pleased to hear Dr. Ward is going to do very specialized work in characterizing what type of kidney lesion this is. Jerry, did you think there was any difference at all or do you even want to comment on the kidneys you were seeing this morning. We saw a lot of kidneys.

Dr. Jerry Ward (No. 605). I think Dr. Klein Szanto showed a very low incidence of amyloid in your untreated two-year-old animals. Right?.

Dr. Andres Klein-Szanto (No. 608). None. Yes, low in the kidney but very high in the liver and spleen.

Dr. Jerry Ward - I thought it was 10 percent.

Dr. Andres Klein-Szanto. No, it was higher than that - around 30 percent.

Dr. Jerry Ward. Yes, but in our animals I saw two cases of liver and spleen amyloid and intestinal amyloid in two out of 30 mice treated with TPA for 2 years. But otherwise, amyloid in the liver and spleen and gut is extremely rare.

Dr. Claudio J. Conti (No. 620). We had about 10 percent of the liver and spleen, but there were of the very old animals and then probably from the population that was living at that time was much higher, 20 or 30 of the population was alive over 2 years.

Dr. Bhalcandra Diwan. I know it appeared as an enigma to have the activation to be effective, but it is degraded or inactivated. Now are the strain differences in response to tumor promotion by TPA related to differences in the degradation of TPA? Does anybody know about it?

Dr. Richard J. Bull. I don't think we have an answer for your question. That means I suspect that it has uniformly not been looked at.

Dr. Bhalcandra Diwan. Okay, my second question to Dr. Hennings. If you take normal epithelial cells, and your initiator cells and then you feed them with NQO or an irritant, biomolecular how are they different? Do they react differently, for example in traction with DNA or even metabolism of those components?

Dr. Henry Hennings. Are you talking about doing that in culture?

Dr. Bhalcandra Diwan: Yes. In culture.

Dr. Henry Hennings. We haven't done that yet. That is something we would like to do. We would like to set up a malignant conversion assay in cell culture, but we haven't yet done that.

Dr. Bhalcandra Diwan. In Vivo? I mean by chemical? Have you done any of that?

Dr. Henry Hennings. We haven't done anything on DNA binding of HQO. No.

Dr. Claudio J. Conti (No. 40). One of topics that maybe was not touched in this meeting was we charted the baseline of immunology in these Sencar mice. Does anybody have any experience of immunology in Sencar mice?

Dr. James Strickland (No. 44). I reported some data on the epidermal Langerhans' cells as you remember. We looked at the numbers of epidermal Langerhans' cells and fresh epidermal sheets of BALB and Sencar by three different techniques: by ATPA staining, IA antigen, and FC receptors - and found that they were indistinguishable between BALB and Sencar. We looked at functional assays for Langerhans' cells which are ala-antigen presenting ability and contact hypersensitivity and again, these were in vivo experiments with adult animals, BALB and Sencar, and found that those were also indistinguishable. And our laboratory, as you remember, has done the skin graft experiments in which Sencar skin and BALB/c skin grafted to nude mouse recipients were treated with DNBA initiation and TPA promotion and the Sencar grafts retained the sensitivity and the BALB grafts retained their resistance, although the sensitivity level of the Sencar was slightly lower in the graft than it was in the intact animal. So our position would be that we have examined these immunological components and the graft experiments indicate the sensitivity as a property of the skin and the particular immune components of the skin which we have looked at and are the major ones that we are aware of, indicate no immunological differences between BALB and Sencar.

Dr. Andres Klein-Szanto (No. 63). How about the humoral immunological parameters. Did anybody look at that? Because these are all cellular-related immunological parameters?

Dr. Claudio J. Conti (No. 67). That was my following comment, but one more. I think some data originated in Cincinnati of different populations of lymphocytes with stimulation of TPA in Sencar mice. Do you remember who's that is?

Dr. Richard J. Bull (No. 70). Yes, Sue Baxter's group here in Cincinnati studies that.

Dr. Claudio J. Conti (No. 71). Is anyone here from this group or section?

Dr. Richard J. Bull (No. 72). Not that I'm aware of.

Dr. Andres Klein-Szanto (No. 73). That's also mainly related to cellular immunological pathways.

Dr. Richard J. Bull (No. 75). To come back to one of the questions that we habe to deal with to some extent. I only heard two possible bases for which there was any evidence that there was a difference between Sencar and other strains that might account for at least their sensitivity to initiators and one had - maybe I shouldn't restrict myself - one had to do with the population of calcium and TPA and sensitive populations to differntiation initiated by calcium or TPA and the other had to do with metabolic differences that Bill Baird was able to identify with the PAH's in his experiments. Do you think either or both of these - how completely do you think Bill's has acounted for differences in sensitivity. That's a teriible question, but, is there obviously something else, tat's the question I'm trying to get around to.

Dr. James Strickland (No. 91). My feeling is that the sensitivity question in Sencar is probably due to more than one factor and we shouldn't be too simplistic about it. However, I think there's certainly a metabolic component. I think the recent paper from John D. Giovanni in which he reported that Dba 2 mice are equally as sensitive as Sencar to initiation and promotion by the directly acting carcingen MNNG and TPA promotion. The data from that paper in which DMBA was used as an initiator indicate that Sencar is more sensitive than Dba 2. My feeling is, although I haven't done any of the studies myself, that Sencar is a pretty good activator of carcinogens that are not presented in their active form. My feeling, based on the data I presented, is that if you present a carcinogen in its active form to the basal cells, which are the target cells, that Sencar and resistant strains will be equally well initiated. There's some practical problems when one is doing in vivo experiments. The target cell is the basal cell, which is the bottom of the epidermis. The carcinogen must get through all the upper layers of the epidermis. There may be some differences between strains and the ability of a given carcinogen to reach the basal target cell and those are things that are fairly simple minded, but they shouldn't be forgotten about. My feeling is that Sencar cells can activate certain carcinogens better than other more resistant strains. If you give the target cells the active form of the carcinogen, Sencar cells are no more sensitive to initiation than other mouse strains, except that the Sencar-initiated cells may have a different sensitivity to promoters than others, so in terms of the number of initiated cells, I would think they would be equivalent, but perhaps qualitatively, the type of initiated cell one gets in terms of response to promoter may be different.

Dr. Gary P. Carlson (No. 122). I showed you half of my slides yesterday. The other half are all the same but they're BALB/c. These papers are all coming out in press. It's a little tough to get them published because they're all negative. Essentially what we show is that the distribution and binding are very similar between the BALB/c and the Sencar. What we do see different is in the amount of material that's present in the tissues, including skin, or bound to DNA in the tissues. The BALB, if anything, shows a higher degree rather than a lesser degree, which, of course, doesn't agree with the tumorigenicity data. But Dr. Strickland's right. If you're measuring binding to DNA and the skin, you're measuring binding to a whole group of cells, so it's difficult to determine what it measn. But there is no simple explanation pharmacokinetically or activation-wise as far as we can tell for the two strains.

Dr. William Baird (No. 135). I think I'd stick my neck out a little further and say that I'm not convinced that that's really the casee yet. I think we see some very small differrences in cell metabolism, Dick. I don't think they could explain the difference, but remember, we've worked wth benzpyrene, and with benzpyrene you see a lot less difference than you do with the DMBA. Now DMBA is a lot tougher compound to work with. Basically, the chemically reactive materials we need to indentify individual DNA adducts have not yet been prepared. They're very difficult to synthesize. The levels are much lower. to give you an example, in the case of benzypyrene, one of these diolepoxide enantiomers is much more active. and yet we have no idea if that exists for DMBA. We don't know whether certain adducts with DNA are critical like they are with methylating agents. I think you really have to get down to what these mechanisms are first. I would say that Gary now has the data that says, okay, it's not going to be a simple problem, the Sencar bind, the BALB/c don't. What we need to do now is to get further into what actually happens and try to relate some of these specific lesions to the carcinogenic process. And then I think you'll begin to find some of the thingss Dr. Strickland is talking about; there are some actual differences between the strains.

Dr. Richard J. Bull (152). Any other comments on tat point? Does anybody want to move on to another point...I thinkl I'm starting to exhaust the things I had written down over the last couple of days. I'm sure there are other things I've missed, that are probably as important or more important.

Dr. William Baird (No. 158). Just to pose a question for you. I guess one of the things that concerns me is the statistical analysis in setting these up for these types of assays. I guess I'd like some comments on, if you were going out to do this particular assay now and you wanted to do it on a reasonable number, other than consulting a statistician, what could we presumably try to do in setting up experiments to make them work very well. What do we really need to set up in order to get a good statistical analysis of the data?

Dr. Richard J. Bull (No. 166). Fortunately, I'm not a statistician. There's a statistician sitting on the end of the table. I think that's a real good question because I don't think the evaluation of responses - I think marginal response has been particularly well done in the past. People are starting to look at what Dr. Nesnow mentioned, some of the methods that they use there, and Dr. Stober talked about some of the problems. It is true unless the statistical approach gets to be standardized and simplified so people understand and they're able to deal with statistics, it's going to be difficult to handle. Judy, do you have any comments about that?

Dr. Judy A. Stober (No. 176). I think the first pl [illegible] where you want to start is to look at all the control data you ha [illegible] see what kind of information is available on the spontaneous rate. The second thing is to standardize how we're calculating responses. The power of the statistical analysis is going to directly depend on how you calculate the response and the number of animals used. If you're going to do a study and you're not going to have a mortality problem, that's one thing. If you're going to do a study where you're going to lose a lot of animals, which may be directly related to the toxicity of your compound, you're going to need a lot more animals to start out to pick up any kind of marginal response at one given point in time. When I looked at the papers that were presented here, I had a great deal of difficulty in comparing those to the type of things that we have done. We usually use 40 animals per treatment group, which is a fair size to start out with. For example, on Dick's slide yesterday he showed a marginal response for FANFT. If you recall, the response curve showed an increase only for the low dose group. The methods used to analyze the data showed a highly significant response rate. Those of you that have shown high responses, i.e. a very high increase in incidence or the average number of tumors per group, I don't recall how many animals you were using in the studies. Therefore, I cannot make a comparison. Additionally I think it is necessary to go back and examine the control data you've used before in your lab and see if the criteria over time has changed for judging responses. If it has then that needs to be taken into consideration. If criteria aren't standardized, it's very hard to predict how to design a good study.

Dr. Richard J. Bull (No. 221). Almost in a way, the problem, in my way of thinking, I can say this now that I'm outside of a regulatory agency, that's almost more of a problem for a regulatory agency because they have to live with what they call positives and negatives, and I think uniformly, with possible exception with what Steve did, everybody else I presented was dealing with tumor responses that you wouldn't question, 5-10 tumors per animal. Assuming most people have similar controls to what we have in terms of incidence. That's not too hard to deal with, even without statistics, but it really becomes a problem, and that's the reason I dwelled on that so much the other day with FANFT, that's absolutely, as she mentioned, highly significantly. Now, for the purposes that we were doing it, I would say I would not depend on that assay to pick up aromatic amines, so the statistic maybe doesn't make any difference because of the low response and the fact that I had some variability in the background, but if you were applying that to a test of an unknown compound, that's a different question. And an agency was gearing up like they were on the diesel thing where they had to do all the experimentation in a very prescribed time, which partly the reason you did that was because they didn't have time to run a 2-year study, they wanted to make a decision quicker than that. You start to get those marginal responses and that's where you really start having problems, I think, because if you do call it a positive, you're stuck with the idea that it's a positive or vice versa.

Are there any questions from the audience because I see Dr. Knutsen going for the microphone.

Dr. Gary L. Knutsen (No. 237). A number of people in the audience during breaks, 4 or 5, asked me during the reception hour last night about the Strain A. We're not done with the Sencar, believe me, but I'm going to get to my point. We're looking at this Sencar and again taking into consideration Dr. Strickland's earlier remarks, we're concerned about the Sencar and skin and we fell upon the Sencar lung and I think I'd like to look at it more. I don't know if we will or not. I've noticed in the literature that now it's very well established that this two-stage carcinogenesis is popping up in other systems, tissue systems, esophagus, colon, bladder, liver, very well documented in the liver. One of the purposes I think here of what EPA is trying to get at and what other people are expressing is that we'd like to look at a number of substances as quickly as possible as a screening agent, picking out the bad actors or the marginal actors and then you can do whole-life studies if necessary. But just as a general question to the toxicologists and other investigators in the audience, what other short-term test should one consider? Are there things on the horizon that should be considered at this time, maybe the Sencar skin isn't the best? Any comments on this?

Dr. Richard J. Bull (No. 267). I think it's got definite limitations as far as being a screening system because ideally a screening system is going to pick up every carcinogen there is. And I do not think it's going to be used in isolation and that's probably true of some of the other systems you made mention of as well. I haven't seen the data on those other systems, I'm not as familiar with them as I am with what we've done with the Sencar and what other people have done with mouse skin in general. Does anybody else have an opinion to offer?

Dr. Gary L. Knutsen (No. 275). It gets back to 1981 when you had the conference here in Cincinnati, first reviewing the feasibility of the matrix and the use of the short-terms. You identified the Strain A lung adenoma, the Sencar papilloma assay, and the liver foci. That's 4-5 years ago. I don't believe there's been another short-term bioassay identified. Has there?

Dr. Richard J. Bull (No. 282). There's the mammary gland, one that's had some attention, and I know that they were doing some evaluations of that at the NCTR, I don't know to what extent they were happy with that. It's a little harder to score and deal with. I don't know, I can't say much more about it than that.

Dr. Stephen Nesnow (No. 289). Well, there is one I just thought of. Paul Netashimon and his co-workers at Oak Ridge and now at NIHS have developed, I think they call it the rat trachea ectopic transplant, where the tracheas are transplanted ectopically to the backs of rats and then they're treated, epithelium is denuded and then they put it into culture and they can look at growth variants, sort of an in vitro/in vivo type assay and it seems to work well for the standard carcinogens, not exactly in the same realm of what you're...

Dr. Richard J. Bull (No. 312). I think, unless there are some burning issues at the table, we've kind of run out of steam here a little bit and it's time to close, but I would like to acknowledge the fact of the organization - and thank Merrel Robinson for organizing this symposium. I think it's the first time we've gotten together in the country. Most of the people who have had interest in the Sencar mouse from one particular viewpoint or another, I hope as the mouse continues to be used - I heard one thing that was mentioned by Dr. Klein-Szanto that the mouse is a very healthy, hardy animals and that it compares favorably in life span to other animals, I think that's one of the big advantages of it that tends to be overlooked. I think that the skin's not only sensitive to carcinogens, it's a good animal to work with which can't be said for all the mice that one would like to deal with in chemical carcinogenesis.

But to thank Merrel Robinson and the Eastern Research Group for arranging the organization of the meeting and I think they did a very good job and I would like to ask that you would give them both a round of applause.